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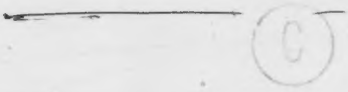
Functional Adaptation of Skeletal Muscle Sarcoplasmic Reticulum in Response to Long-Term, High-Intensity Endurance Exercise



by

Siobhann Marie Williamson

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE

OF MASTER OF SCIENCE

Department of Physical Education and Sport Studies

Edmonton, Alberta

Fall, 1987

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PERMANENT ADDRESS:

c/o 402 - 1865 Glenanna Rd

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Functional Adaptation of Skeletal Muscle Sarcoplasmic Reticulum in Response to Long-Term High-Intensity Endurance Exercise submitted by Siobhann Marie Williamson in partial fulfilment of the requirements for the degree of Master of Science.

A Belcastro

SUPERVISOR

Date: 26 August 1987

This thesis is dedicated to

MUM and DAD

who have always always encouraged my brothers and me

to strive for excellence

ABSTRACT

The effects of long-term, high intensity endurance exercise were examined in purified light and heavy vesicles of sarcoplasmic reticulum isolated from the superficial vastus lateralis muscle of female adult rats. Light and heavy vesicles of control tissue were typical in terms of peptide profile, ratio of 115 kdal to 30 kdal proteins and Ca^{2+} ATPase activity. Training induced adaptation of the SR (ie. yield of membrane protein, Ca^{2+} ATPase activity, peptide profile and the ratio of the 115 kdal to the 30 kdal proteins) was limited to the vesicles isolated from the terminal cisternae (heavy) membranes, suggesting a separate gene for the production of the Ca^{2+} ATPase or different regulation of the same gene in these two regions of the SR. The fact that defined parameters changed in the same direction and with at least the same magnitude as those observed in previous studies employing this training regimen confirms that a training program is capable of inducing transformations of the functional characteristics of skeletal muscle. A secondary objective of the study was to observe growth rates and food consumption in sedentary and exercise groups throughout the program. The rats of the training group consistently consumed less chow than their sedentary counterparts but at no time was there a statistical difference in body mass between groups.

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I wish to acknowledge the generous contributions of my committee members, Dr Vickie Baracos and Dr Art Quinney and that of my advisor, Dr Angelo Belcastro.

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LIST OF ABBREVIATIONS

ES	electrical stimulation
PT	physical training
HR	heart rate
SV	stroke volume
MZ	monozygotic
DZ	dizygotic
DH	dehydrogenase
SR	sarcoplasmic reticulum
Me His	methylhistidine
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
RR	ruthenium red
NaN_3	sodium azide
ATP	adenosine triphosphate
ADP	adenosine diphosphate
ESR	electron spin resonance
LDH	lactate dehydrogenase
FSR	fractionated sarcoplasmic reticulum
BSA	bovine serum albumin

CHAPTER 1

Introduction

The existence of skeletal cell muscle types which vary in their contractile, metabolic and functional characteristics enable the mammal to perform a variety of endurance, power and skill activities. The motor unit (the collective term for the motor neuron and the muscle fibres which it innervates) are under control of the nervous system. The physiological parameters appear to be genetically determined but vary somewhat in response to use.

Fibre-specific differences have been documented and may be divided into contractile, metabolic and functional domains. Table 1.1 summarizes these fibre specific characteristics and a detailed discussion of the fibre-specific differences is located in Appendix A. Speed of contraction is proportional to myosin ATPase activity. The existence of polymorphic forms of this contractile protein allows for the division of skeletal muscle into two well defined groups. *In vitro* characterization is made possible due to the fibre specific differences in the sensitivity of this ATPase to activation or inactivation in response to pre-incubation in acid or alkaline solutions (Brooke and Kaiser, 1969). The so-named type I fibres appear lightly stained (Engel, 1962) and have relatively long peak contraction and relaxation times

(Buchthal and Schmalbruch, 1970), thus the designation slow twitch fibre. Likewise, fibres which are darkly stained and have comparatively short times to peak contraction and relaxation are known as type II, fast twitch muscle fibres.

Metabolic properties have allowed classification of fibre types which are not only consistent with, but further extend that achieved through contractile characterization. In vitro histochemical techniques are based on the variability of the concentration of glycolytic and oxidative enzymes. The type I (slow type) fibres have been shown to contain a high concentration of enzymes of the citric acid cycle but were limited in their ability to convert carbohydrate to lactate (Haggmark and Thorstensson, 1979). In contrast are the type II (fast type muscle), where the enzyme profile is not as distinct. Some muscles found to stain dark after myofibrillar ATPase staining possess the anticipated enzyme profile, that is high glycolytic and low oxidative capacities. However, another subpopulation was observed which was well endowed in both enzyme systems. The nomenclature thus assigned to the high oxidative high glycolytic fibre was type IIA while those fibres high in glycolytic enzymes but low in oxidative enzymes were named type IIB (Barnard et al., 1971). While considerable variability in enzyme characteristics may exist within a designated fibre type (Lowry et al., 1978), limited variation occurs in fibres within a motor unit (Pette et al., 1980).

TABLE 1.1: A summary of relative fibre specific characteristics of skeletal muscle in untrained humans (adapted from Saltin et al., 1977).

	FIBRE		
	Type I	Type IIA	Type IIB
Fibre Area	++	+++	+
Capillary Supply	+++	++	+
Myosin ATPase activity	+	(+++)	
Time to Peak Tension	+++	(+)	
Glycolytic Potential	++	+++	++++
Oxidative Potential	+++	++	+
Glycogen Storage Potential	+++	+++	+++
Triglyceride Storage Potential	+++	+	+

Legend: low + ++ +++ ++++ high

Functionally, it is of interest to examine the fibre specific parameters of the sarcoplasmic reticulum (SR), the subcellular organelle responsible for calcium sesquestration from the contractile machinery and hence relaxation. Sembrowich et al., (1985) noted a greater yield of SR from type II fibres than from type I fibres, some researchers reporting up to two-fold difference (Feihn and Peter, 1971; Wang et al., 1979). Protein composition of the SR from different fibre types revealed fibre-specific differences only in the number and mobility of minor protein components, of lower molecular weight (Margreth et al., 1974b). This group reported a reduced amount of the 100 kdalton protein (identified as the Ca^{2+} stimulated-Mg^{2+} dependent ATPase (MacLennan et al., 1971)) when compared to type II fibres. It has been documented that the highest concentration of the Ca^{2+} ATPase and a membrane bound calcium binding protein, calsequestrin, is observed in type IIB fibres (Zubrzycka-Gaarn et al., 1982; Wang et al., 1979). The ratio of the 100 kdal protein (the Ca^{2+} ATPase) to the 30 kdal flavoprotein (NADH cytochrome b_5 reductase (Salviati et al., 1981)), has been proposed as a fibre-specific marker (Weihrer and Pette, 1983). In vitro simulation of the action of the Ca^{2+} ATPase is achieved through the use of vesicles of fragmented SR (FSR) isolated through differential centrifugation. In type I fibres, SR was observed to play a negligable role in calcium movements linked to relaxation (Margreth et al.,

1974a) as the mitochondria was observed to take part in calcium uptake. This group observed that the initial rate of Ca^{2+} accumulation in type IIA fibres was as much as 10 times that recorded for type I fibres. Many investigators have concluded that the maximum rate of uptake was at least 4 times higher in type IIB than type I fibres (Feihn and Peter, 1971; Kim et al., 1981). This is thought to be more than just a function of the number of active sites as Wang et al., (1979) observed variation between fibre types with reference to the affinity of the Ca^{2+} ATPase for calcium.

Variation is also noted within the SR, between portions of the membrane derived from distinct regions. The terminal cisternae (that area of the SR which is in immediate contact with the t-tubules) may be differentiated from the longitudinal reticulum (that area of the membrane which forms an anastomosing network overlying the A-band region of the sarcomere), by virtue of the presence, in the former region, of the calcium binding protein, calsequestrin (Campbell et al., 1980). Based on migration in a sucrose density gradient, the former region is termed "heavy SR" and the latter "light SR" (Lau et al., 1977).

The functional consequences of these fibre specific characteristics are such that the type IIB fibres are poorly designed to maintain long-term aerobic metabolism in comparison to type I fibres. The type IIb fibre is associated with a high speed of contractile activity and a

low resistance to fatigue.

Klissouras (1976) reported on a study which utilized percutaneous muscle biopsy to examine some ultrastructural characteristics of human skeletal muscle in monozygotic (MZ) and dizygotie (DZ) twin pairs. Since they observed no intrapair differences for selected well-defined ultrastructural and metabolic parameters, it was concluded that any interindividual variability arose from extragenetic influences. However, Komi et al., (1977) observed an almost identical percentage of type I skeletal fibres in the vastus lateralis muscles of MZ twins and not in DZ twins. Fugl-meyer et al., (1982) observed similar genetic relationships in fibre type composition of human forearm muscles. It has thus been proposed that the motor unit composition in skeletal muscle is genetically fixed such that a motor neuron innervates a physiologically uniform fibre type and that this fact has implications upon potential for physical performance.

Electrical stimulation (ES) has been used as a model to examine the neural control of gene expression in adult skeletal muscle. Appendix A contains a lengthy discussion of the effects of ES. Chronic indirect electrical stimulation of type II fibres at a frequency pattern resembling that which occurs naturally in slow-twitch type I muscle has been used to induce a fibre-type transformation. These protocols have been especially useful because the mechanics of the

motor unit can be amplified and because the procedure may be examined in "slow motion" through the application of intermittent protocols. Changes induced by ES have been well documented in the main areas of muscle function, namely energy metabolism (Table 1.2), SR function (Table 1.3), function of the myofibrillar proteins (Table 1.4) and ultrastructure (Table 1.4). Recent work has demonstrated that the frequency pattern is not the major influencing factor, but rather the total amount of contractile activity induced upon the muscle (Sreter et al., 1982). This leads one to speculate whether a load of contractile activity, imposed upon the muscle in what may be considered as a more physiological setting, such as exercise, may achieve comparable transitions of the muscle fibres metabolic, contractile and functional characteristics. To date, the bulk of research using training protocols have been unsuccessful at achieving more than metabolic and limited functional changes in response to increased contractile activity. It may be suggested however, that the exercise regimes used may have been too weak to elicit transformational changes or that indeed "complete" transformation of fibre type may not be viable at this level.

Green et al., (1983) using an intense long term endurance running protocol observed changes in the enzyme profile which were quantitatively similar to those observed in studies using electrical stimulation. After the same

TABLE 1.2: Summary of contractile changes in animal skeletal muscle induced by electrical stimulation (ES) or physical training (PT). (See also Appendix A).

PARAMETER	ES	Source	PT	Source
Time to peak tension	↓	20,21,22,23		
Time to 1/2 relaxation	↓	22,24,25,26		
Twitch tension ratio	↓	24		
Myosin ATPase activity	↓	18,20,27	○	50,28,29,30
Light chains:				
1f, 2f, 3f	↓	31,32,33,34		49 (1f,3 of IIA)
1s, 1s',2s	↓	28,29,30,	○	49 (1s,2s)
Myosin heavy chains (ie. N⁻ MeHis)	↓	29		
Tropomyosin	↓	31,37		
Mg^{2+} myofibrillar ATPase Activity	↓	20	○	48

Note. "Source" numbers correspond to the numbered references which appear at the end of this chapter.

TABLE 1.3: Summary of metabolic changes in animal skeletal muscle induced by electrical stimulation (ES) or physcial training (PT). (See also Appendix A).

PARAMETERS	ES	Source	PT	Source
Hexokinase	↑	1,2,4,7,8		
Pyruvate Kinase	↑	2,7	↔	46,47
Phosphofructokinase	↑	2,7	NC	47
Lactate Dehydrogenase (DH)	↓	1,2,5,7	↔ A B NC	46
Succinic Dehydrogenase	↑	1,2,3,4		
Glycerophosphate DH	↓	1,2		
Citrate Synthase	↑	2,4,5,6,7	↔	46,47
Fructose-1,6-Diphosphatase	↓	2,7	↓	47
Glycogen Phosphorylase	↓	1,2,5	↓ A B NC	47
3-Hydroxy-CoA-DH	↑	1,2,4, 5,6,7	↔	47
Palmitoyl-CoA-Synthase	↑	39		
3-Ketoacid Transferase	↑	1	↔	47
B-Hydroxybutyrate DH	↑	4		
Creatine Kinase	↓	1		
Adenylate Kinase	↓	1		

Note. "Source" numbers correspond to the numbered references which appear at the end of this chapter.

TABLE 1.4: Summary of functional (SR) changes in animal skeletal muscle induced by electrical stimulation (ES) or physical training (PT). (See also Appendix A).

PARAMETER	ES	Source	PT		Source
			IIA	IIB	
SR Yield	↑	13	NC	NC	48
Parvalbumin content	↓	9,14	↓	↓	49
Ca^{2+} ATPase Activity	↓	15			
Initial rate of Ca^{2+} uptake	↓	13,16			
Total Ca^{2+} uptake	↓	7,13,16			
	↓	17,18	↑		48
[115 kdal] protein	↓	13,15,16	↓	↓	49
[30 kdal] protein	↑	15,16	↑	↑	49
ratio 115 kdal / 30 kdal	↓	19	↓	↓	49
7-9 nm particles	↓	16			
concave:convex (A:B ratio)	↓	16			

Note. "Source" numbers correspond to the numbered references which appear at the end of this chapter.

TABLE 1.5: Summary of ultrastructural changes in animal skeletal muscle induced by electrical stimulation (ES) or physical training (PT). (See also Appendix A).

PARAMETER	ES	Source	PT	Source
Capillarization	↑	4,38,40	⌂	54,55
Fibre x-sec. area	↓	14,38,40,41	NC	55
Yield of t-system	↓	41		
Mitochondrial content	↑	4	⌂	52,55
Unit volume of mitochondrial membrane	↓	4		
Sarcolemmal mitochondria	NC	4	⌂	56,55
Intracellular Lipid	NC	4	⌂	55
Z-disc width	↑	41,43,44		

Note. "Source" numbers corresponds to the numbered references which appear at the end of this chapter.

experiments, Green et al., (1984) reported changes in the myosin isozyme profile of the type IIa fibres, in the same manner, but to a lesser degree as those documented after electrical stimulation.

Functional changes documented by Green et al., (1984) included decreased concentration of the cytosolic Ca^{2+} binding protein, parvalbumin, and changes in the electrophoretic pattern of some SR membrane proteins. A decrease in the concentration of the Ca^{2+} ATPase, in type II fibres and an increase in the concentration of 30 kdal NADH cytochrome b^5 reductase lead to a decrease in the ratio of these proteins, a phenomenon observed after electrical stimulation.

It therefore appears that some changes in the SR protein ratio can occur through an exercise stimulus but the extent to which these protein changes represent Ca^{2+} transport changes is unknown. Similarly, there is no literature available as to the adaptability of the SR derived from different regions of the membrane. On this basis, the following questions were proposed:

1. Can high intensity endurance training induce a change in the SR Ca^{2+} transport profile of type IIB fast twitch glycolytic fibres (ie. yield of sarcoplasmic reticululm, Ca^{2+} ATPase activity, and the fibre specific ratio of select membrane proteins) such that it resembles the previously defined characteristics of type I slow twitch oxidative

fibres?

2. Is there a difference in the degree to which high intensity endurance training affects the fractions of the sarcoplasmic reticulum derived form the terminal cisternae and the longitudinal membrane?

3. Is there a difference in the quantity of food consummed and the pattern of growth in the animals of the sedentary and exercise groups during a program of high intensity endurance training?

Definitions

Heavy SR is that portion of the membrane which originates from the terminal cisternae and migrates in a density gradient with the isopycnic point at 40% sucrose (Lau et al., 1977).

Light SR is that portion of the membrane which originates from the longitudinal SR and migrates in a density gradient with the isopycnic point at 31% sucrose (Lau et al., 1977).

The Suitability of the Experimental Model

The use of animals to study the effect of high intensity endurance training has several advantages over the use of humans. The documumented fibre interconversions achieved through electrical stimulation have been demonstrated in the rat, rabbit and cat. In order to be able to compare the results of this study with other published work, the rat was chosen. The animal model is well suited to control and manipulation of factors such as environment, diet, drugs and

infectious disease. Unfortunately, in the rat, the potential for motivation is not as great, necessitating the use of negative reinforcement.

For the purpose of studying adaptive responses of skeletal muscle to a training stimulus, the use of the rat model is therefore both appropriate and adequate. However, not all of the responses of the rat to exercise training are identical to those seen in humans which limit their use for certain types of studies. For example, the rat does not experience an increase in stroke volume in response to exercise (Popovic et al., (1969) in Barnard et al., 1974)) therefore an increase in cardiac output is manifested entirely as an increase in heart rate (HR). Thus, Barnard et al., (1974) observed that even at moderate workloads, the steady state HR was above 80% of maximum HR. In addition, they observed that untrained rats which were made to run at 10% grade and 26.6 $m \cdot min^{-1}$, responded with a HR of 95% of maximum. In spite of this apparently excessive rate, these researchers observed that unlike the human, some untrained rats could work at this rate for upwards of 3 hours. It is assumed, however, that these differences do not affect the fundamental ability of skeletal muscle from any species of mammal to adapt to a training regime.

Delimitations

To control for a sex-linked interaction, only female rats were used in this investigation. Oscai et al., (1971)

reported that intense exercise appears to have an appetite supressing effect upon male rats which is superimposed upon the increased caloric expenditure of the exercise regime. They hypothesized that the appetite supression is mediated by stress induced catecholamine release. However, these researchers observed that female rats gained weight at the same rate as their sedentary controls, as a result of a voluntary increase in food consumption beyond that of the sedentary group. They attempt no explaination for this sex difference. While it may be expected that the sex of the animal will not influence its potential for fibre transformation, one sex was used in an attempt to ensure that both control and training groups were healthy from a nutritional standpoint.

The heart and the tibialis anterior, epitrochlearis and soleus muscles were used to identify training-induced changes in muscle mass and total muscle protein, but only the superficial portion of the vastus lateralis muscles were analysed for functional changes of the SR. Baldwin et al., (1972) reported that the superficial portion consisted primarily of type IIB fibres. It may be assumed, therefore, that this portion of the muscle would be the last to be recruited on the intensity-related hierarchy of motor unit recruitment. Any biochemical changes which were observed in this muscle may therefore be observed to occur to at least the same degree in the deep and intermediate portions of the

vastus lateralis. Baldwin et al., (1977) in a time course observation of interval and intense endurance running programs, found that citrate synthase increased in all three fibre types in the vastus lateralis. With the endurance protocol, the change observed in the type IIB fibres regressed as the animal adapted to the stress. However, the interval training protocol had the greatest relative effect upon the type IIB fibres, pointing out the need for a program having both endurance and interval components in order to continually recruit those fibres. Based on this intensity dependent recruitment effect, only superficial vastus lateralis muscle were analysed.

The protein identified throughout the literature as the Ca^{2+} ATPase has associated with it, a range of molecular weights of between 100 kdal and 120 kdal[9]. For the sake of consistency, this protein shall be referred to as having a molecular weight of 115 kdal, in this thesis.

The term "functional" appears throughout this thesis and is used in the context of the function of SR in terms of its calcium translocating abilities. Literature dealing with the SR may therefore be divided into contractile, metabolic and functional data.

References and their numerical assignment, as described in TABLES 1.2 - 1.5 and A-2:

1. Pette et al., 1973
2. Harris et al., 1982
3. Pette and Tyler, 1983
4. Reichmann et al., 1985
5. Pette et al., 1972
6. Buchegger et al., 1984
7. Hudlicka et al., 1984
8. Helig and Pette, 1980
9. Klug et al., 1983a
11. Baldwin et al., 1977
12. Green et al., 1983
13. Heilman and Pette, 1979
14. Pette et al., 1985b
15. Klug et al., 1983b
16. Heilman et al., 1981
17. Ramirez and Pette, 1974
18. Sreter et al., 1982
19. Wiehrer and Pette, 1983
20. Buller et al., 1969.
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23. Edwards et al., 1982
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28. Sreter et al., 1973

29. Sreter et al., 1974

30. Sreter et al., 1975

31. Roy et al., 1979

32. Brown et al., 1983

33. Helig et al., 1984

34. Seedorf et al., 1983

35. Sreter et al., 1982

36. Pette and Schnez, 1977

37. Helig and Pette, 1983

38. Brown et al., 1976

40. Hudlicka et al., 1982a

41. Eisenberg and Salmons, 1981

43. Salmons et al., 1978

44. Salmons, 1980

45. Romanul et al., 1974

46. Baldwin et al., 1977

47. Green et al., 1983

48. Belcastro and Wenger, 1982

49. Green et al., 1984

50. Bonner et al., 1976

51. Klug et al., 1983a

52. Gollnick and King, 1969

53. Orlander et al., 1977

54. Ingjer, 1978

55. Hoppeler et al., 1985

CHAPTER II

Methodology

1. Animal Selection

Mature (mean age 75 days, initial body mass 177.2 g), female Sprague Dawley rats of the Buffalo strain (bred at the University of Alberta, department of Animal Science) were used in this investigation.

2. Animal Care

The rats were randomly assigned to either a running group (N=23) or a sedentary control group (N=17). Three weeks prior to the end of the study approximately half of the rats from each group were donated to a separate study. A larger number of animals were assigned to the running group to attempt to achieve a sufficient numbers in light of the possibility of attrition due to significant injury or illness, leaving a final N of 8 and 7 for the control and running groups respectively.

The rats were housed in individual hanging cages outfitted with a metal plate which occupied approximately 1/3 of the floor area to create a smooth surface upon which the animals could rest. They were maintained on a reverse light/dark cycle and were fed water and ground Purina laboratory rat chow ad libitum.

3. Animal Preparation

Prior to each training period, both runner and sedentary

control animals were transported from their storage room to the exercise physiology laboratory in the Surgical Medical Research Institute.

4. Warm-Up and Cool-Down

The rats were placed in the appropriate treadmill channels and the (Quinton Instruments Rodent) treadmill was set in motion. The initial incline was zero degrees. The treadmill speed and incline were gradually increased over 6-8 minutes such that the timer was started when the speed had reached 27 $m \cdot min^{-1}$ and the incline was approximately 75% of the prescribed angle of incline for that particular training day. The incline was gradually increased to the desired degree over the first 2 minutes of the timed run.

When the timed run had been completed, the treadmill angle was immediately decreased to zero. The animals continued to run for 2-3 minutes as the belt was gradually brought to a stop. While Barnard et al., (1974) report that, in the rat there is a very rapid drop in recovery heart rate within the first minute, post exercise, with no further change in HR in the subsequent 10 minutes, our protocol was adopted to aid venous return after one animal appeared to experience post-exercise cardiac arrest resulting in death.

5. Training Program

The training program employed a 15 week, 6 $days \cdot week^{-1}$ program which involved increases in both duration (minutes) and angle of incline (degrees). It was an adaptation of the

program designed by Green et al., (1983) from Terjung (1976). (Table 2.1)

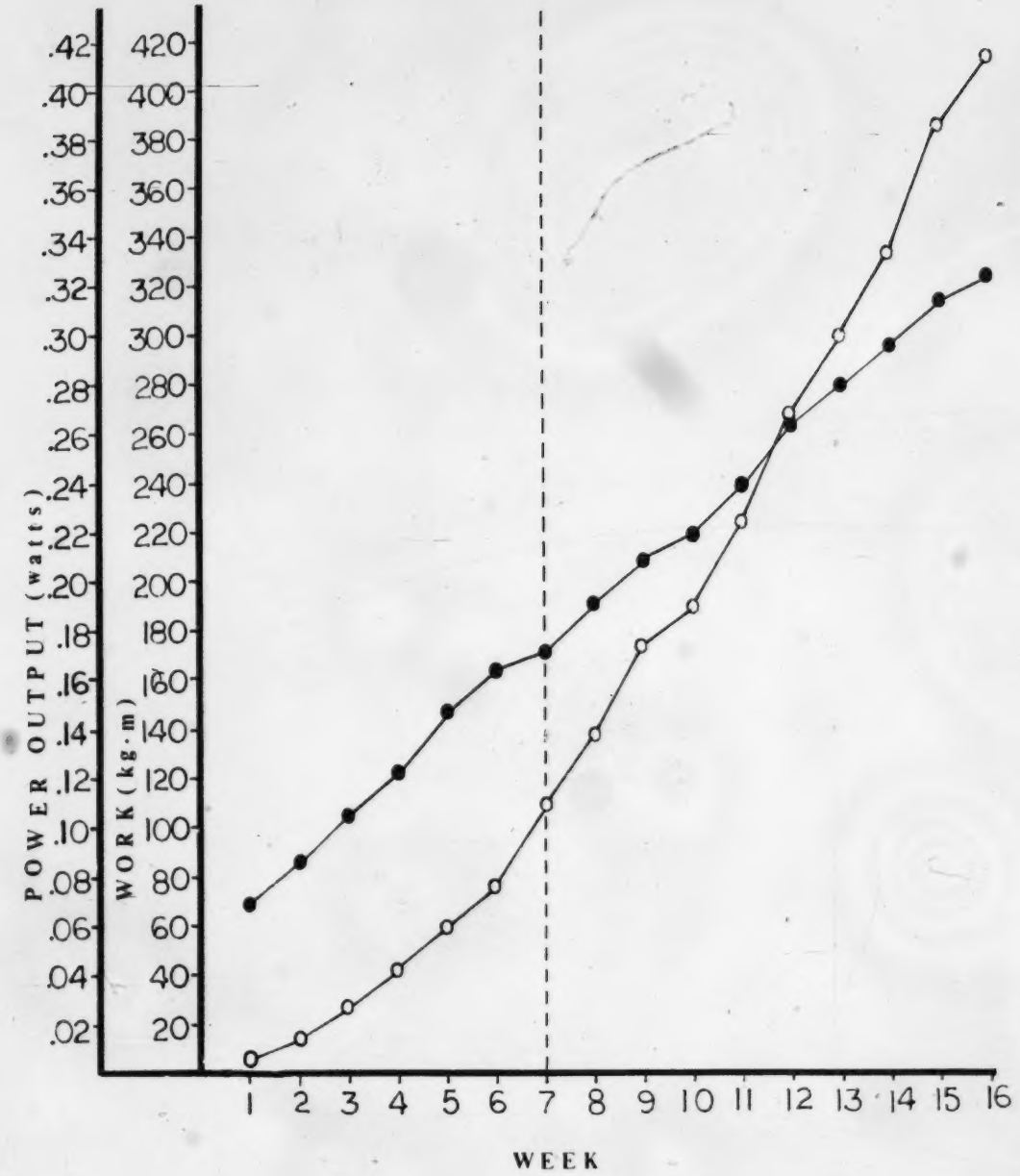
The running speed was maintained at 27 $m \cdot min^{-1}$. Beginning at 12 $mins \cdot day^{-1}$ and 5 degrees, the duration and angle of incline were increased by 2 minutes daily and 0.5 degrees every 3 days, respectively. It is apparent that Green et al., (1983) occasionally had to make adjustments to the progression in intensity and duration from their original schedule, due to the inability of the rats to complete the work session. The present study employed a program which was designed to meet the same weekly and final work loads as the 1983 study but an incremental adjustment was made to the design, such that the duration of each bout and the angle of incline were increased in smaller increments. While Green et al., (1983) increased the time by 3 minutes daily and the incline by 0.5 degrees every two days, the program in the present study employed increases of duration of 2 minutes daily and 0.5° every 3 days. To ensure that work load and power output increased gradually but significantly throughout the duration of the study, these parameters were calculated and monitored daily (Figure 2.1).

As in the study by Green et al., (1983), the daily exercise was split into two sessions at week 7, since previous research has linked exercise duration and glycogen depletion. Terjung et al., (1974) noted that glycogen levels in fast twitch glycolytic fibres and slow twitch oxidative fibres

TABLE 2.1: Training Protocol

WEEK	TIME (minutes) / ANGLE OF INCLINE (degrees) DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6
1	12 / 5	14 / 5	16 / 5	18 / 5.5	20 / 5.5	22 / 5.5
2	24 / 6	26 / 6	28 / 6	30 / 6.5	32 / 6.5	34 / 6.5
3	36 / 7	38 / 7	40 / 7	42 / 7.5	44 / 7.5	46 / 7.5
4	48 / 8	50 / 8	52 / 8	54 / 8.5	56 / 8.5	58 / 8.5
5	60 / 9	62 / 9	64 / 9	66 / 9.5	68 / 9.5	70 / 9.5
6	72 /10	74 /10	76 /10	78 /10.5	80 /10.5	82 /10.5
7	84 /10.5	86 /10.5	88 /10.5	90 /10.5	92 /10.5	94 /10.5
	12 /10.5	14 /10.5	16 /10.5	18 /10.5	20 /10.5	22 /10.5
8	94 /11	94 /11	94 /11	94 /11.5	94 /11.5	94 /11.5
	24 /11	26 /11	28 /11	30 /11.5	32 /11.5	34 /11.5
9	94 /12	94 /12	94 /12	94 /12.5	94 /12.5	94 /12.5
	36 /12	38 /12	38 /12	40 /12.5	42 /12.5	44 /12.5
10	94 /13	94 /13	94 /13	94 /13.5	94 /13.5	94 /13.5
	48 /13	50 /13	52 /13	54 /13.5	56 /13.5	58 /13.5
11	94 /14	94 /14	94 /14	94 /14.5	94 /14.5	94 /14.5
	60 /14	62 /14	64 /14	66 /14.5	68 /14.5	70 /14.5
12	94 /15	94 /15	94 /15	94 /15.5	94 /15.5	94 /15.5
	72 /15	74 /15	76 /15	78 /15.5	80 /15.5	82 /15.5
13	94 /16	94 /16	94 /16	94 /16.5	94 /16.5	94 /16.5
	84 /16	86 /16	88 /16	90 /16.5	92 /16.5	94 /16.5
14	94 /17	94 /17	94 /17	94 /17.5	94 /17.5	94 /17.5
	96 /17	98 /17	100/17	102/17.5	104/17.5	106/17.5
15	94 /18	94 /18	94 /18	94 /18.5	94 /18.5	94 /18.5
	108/18	110/18	112/18	114/18.5	116/18.5	118/18.5

FIGURE 2.1: Work Load (●——●) and Power Output (○——○) calculated on the final training day of each week. The protocol consisted of daily bouts of exercise at 27 $m \cdot min^{-1}$, beginning at an incline of 5 degrees for a duration of 12 minutes. The duration was increased by 2 min every day and the incline was increased by 0.5 degrees every 3 training days. At week 7, the training sessions were held twice each day (— — —).


POWER OUTPUT (watts)
.42
.40
.38
.36
.34
.32
.30
.28
.26
.24
.22
.20
.18
.16
.14
.12
.10
.08
.06
.04
.02
WORK (kg·m)
420
400
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
WEEK

were fully repleted 4 hours after an exhaustive run, while the fast twitch glycolytic fibres had only recovered 75% of the muscle glycogen. However, with training, enzymes specific to glycogen repletion (hexokinase and glycogen synthetase) increase (Terjung et al.,(1974); Baldwin et al., (1977); Green et al., (1983)) so it may be expected that muscle glycogen is restored after 6 hours. Thus, for the first 6 weeks, the rats ran 6 days·week^{-1} at 8 a.m.. From weeks 7 to 15, the rats trained at 8 a.m. and again at 6 p.m. The duration of the morning run was maintained at the 94 minutes which had been achieved by the end of the 6th week; the evening run began at 12 minutes and increased by two minutes·day^{-1}. Both daily bouts followed the progression in treadmill angle. The duration of the run on the final training day was a total of 212 minutes at an incline of 18.5 degrees. A mechanical dysfunction in week 9 lead to the program being set back by 1/2 week.

6. Post-Mortem Treatment

All rats were sacrificed by injection with sodium pentobarbital 36-48 hours after the final training run, followed by dissection of the vastus lateralis, tibialis anterior, epitrochlearis and soleus muscles and the heart. The muscles were weighed after removal of fascia and adipose tissue. The deep and superficial vastus lateralis were separated by visual appearance. Baldwin et. al., (1972) reported that the former consists primarily of fast oxidative

glycolytic fibres. The muscles were quick frozen in liquid nitrogen using a freeze clamping technique. Samples were stored at -70° C until analysed.

7. Biochemical Analysis

(see Appendix B for details)

Total muscle protein was determined on the right epitrochlearis and tibialis anterior muscles of control and trained animals. Following the proceedure of Lowry et al., (1951; as modified by Campbell and Sargeant, 1967) the whole muscle homogenate was diluted in a suspension buffer of 300 mM sucrose, 5 mM imidazole HCl and 0.5 mM dithiothreitol (DTT) and assayed for total protein concentration against a standard curve of known concentrations of bovine serum albumin (BSA).

Native vesicles of SR were isolated from the superficial vastus lateralis muscles of control and trained animals, by density gradient centrifugation following the proceedure of Saito et al., (1984). Briefly, whole muscle was first ground under liquid nitrogen before being homogenized in a medium of 300 mM sucrose, 20 mM Imidazole HCl, 0.5 mM ethylene diamine tetraacetic acid (EDTA) and 0.5 mM DTT. After a set of differential centrifugation, the purified pellet was layered onto a sucrose density gradient and centrifuged for 16 hours at 20,000 rpm. The second and third fractions, representing light and heavy fractions of SR vesicles respectively, were resuspended in 5 mM imidazole HCl and further purified before

being frozen in a media of 300 mM sucrose, 5.0 mM imidazole HCl and 0.5 mM DTT, for future biochemical analysis. The protein concentration of the solutions of isolated vesicles was determined following the proceedure of Lowry et al., (1951) using a standard curve of known BSA concentrations.

ATPase enzyme activity was determined following a modified version of the proceedure reported by Parkhouse and Belcastro, (1987). Calcium-stimulated, magnesium dependent enzyme activity was determined in the presence of 5 uM Ca^{2+}. Magnesium stimulated ATPase activity was determined in a media which contained 1 mM ethylene glycol tetraacetic acid (EGTA). To ascertain the degree of contamination by mitochondria in the isolated protein preparations, ATPase activity was determined in a solution containing 10 uM sodium azide (NaN_3). A solution containing 15 uM ruthenium red (RR) was prepared to evaluate the enzyme activity in the face of blocked calcium channels. The reactions were carried out in solution which contained one of the above listed constituents along with 2 mM phosphoenolpyruvate (PEP), 0.2 mM reduced nicotinamide adenine dinucleotide (NADH), 10 U lactate dehydrogenase (LDH), 10 U pyruvate kinase (PK), buffered by a standard reaction media which contained 300 mM sucrose, 100 mM KCl, 10 mM piperazine ethane sulfonic acid (PIPES) and 7.5 mM $MgCl_2$ and the homogenization buffer as detailed above. Following a 10 minute incubation at 37·C, the reactions was initiated by the addition of 5 mM MgATP. The reaction was

terminated by an excess of 70% perchloric acid after 10 minutes. Enzyme activity was assessed by calculation of the change in optical density as a function of protein concentration and time. Calcium stimulated ATPase activity was calculated by subtracting that value observed in a medium containing EGTA from that observed in a medium containing free calcium.

Samples of light and heavy SR vesicles from control and trained animals were denatured with sodium dodecylsulfate (SDS) and layered onto a polyacrlyamide gel of 2% stacking and 10% running layers (Laemmli, 1970). The gels were stained with Coomassie Blue. After drying and mounting, the concentration of the separated proteins was determined by densitometry at 540 nm using an LKB Ultroscan. Relative concentration of the 4 major SR membrane proteins was determined by cutting-out and weighting the area under the protein-specific peaks on the densitometric scans.

8. Statistics

(see Appendix C for details)

Body weight and food consumption were analysed with a multivariate ANOVA with repeated measures against time incorporating a Greenhouse-Geiser analysis (Hopkins and Glass, 1978). Muscle mass, heart mass as well as the ratio of heart mass : body mass (HM : BM) were compared between exercise and control groups using a Student's t-test. Total muscle protein, SR yield and ATPase activities were analysed

by a 2-way ANOVA, with a post hoc Neuman Kuel t-test. Ca^{2+}, Mg^{2+} ATPase data were compared to that with sodium azide (NaN_3) and ruthenium red (RR) by Student's t-test.

CHAPTER III

Results

1. Success of the Training Program

The training program employed in the present investigation was a modification of that used by Green et al., (1983 and 1984) as originally designed by Terjung (1976). The running speed was maintained as in the previous reports, however, the total time and angle of incline were somewhat larger than that used earlier. Many of the animals sustained injury such as severe abrasions of the foot and tail from the seam of the tread and muscle sprains from becoming caught between the moving tread and the stationary channel apparatus. This resulted in injured animals being given 2 or 3 days of rest after which time a judgement was made on their recovery, and ability to return to the training program. Total work was made-up where possible. Twenty-five percent of the running group were eliminated due to recurrent injury or the inability to complete the required exercise bout while 13% of the running group were eliminated due to accidental death.

Despite the apparent high rate of attrition, attributable to injury rather than inability to adapt to the cardiovascular stress, it was observed that all healthy rats were quite capable of completing the program, such that in the final training days these rats were running up an incline of 18.5 degrees for over 3.5 hours.

2. Anthropometric Data

Animals of both the control and training groups continued to mature throughout the study. Figure 3.1 represents the weekly mean body mass and food consumption for each group determined on the final training day of each week. At no time was there a statistical difference ($p > .05$) between groups: however, the training group tended towards lower body mass in the final weeks of the study.

Control and endurance trained rats did not differ in their heart mass ($p > .05$) when compared on either an absolute or relative basis the between group difference was still not significant ($p > .05$). Table 3.1 summarizes the final body and tissue weight data. Muscle mass of the epitrochlearis and tibialis anterior muscles did not differ between groups ($p > .05$), although a trend was evident. Normalized muscle mass was not significantly different between groups. Total muscle protein did not differ between groups (Table 3.1).

3. Food Consumption Data

A significant difference between groups in terms of food consumption was not apparent until the 4th week ($p < .05$). For the duration of study, the larger food consumption by the trained rats remained and was not reflected in a larger body mass (Fig. 3.1).

3. Electrophoretic Data

PLATE 3.1 shows SDS PAGE of light and heavy SR vesicles obtained from the superficial vastus lateralis muscles of the control and trained rats. Co-electrophoresis with protein standards (lane C) revealed that the proportions of major SR proteins (namely, Ca^{2+} ATPase, calsequestrin, High Affinity Calcium Binding Protein (HACBP), and the 30 kdal NADH cytochrome b^5 reductase) were typical of that observed in light and heavy fractions of tissue from untrained rats. Quantitative evaluation of the proteins by densitometry demonstrated that 79% of the total protein in light vesicles of control tissue was the Ca^{2+} ATPase. In heavy vesicles, this calcium translocating protein constituted 70% of the total membrane proteins of this fraction. The ratio of the 115 kdal protein and the 30 kdal protein, which has been suggested as a fibre-specific marker (Wiehrer and Pette, 1983) is shown in Table 3.3.

5. Biochemical Data

Total muscle protein was not significantly different between groups or between muscles within a group ($p>.05$; table 3.1). The training protocol had an effect upon SR yield in both vesicle types such that SR isolated from the muscle of trained animals was less abundant ($p<.05$; see Table 3.4). Likewise within either group significantly more SR was obtained from heavy vesicles. The interaction of the

FIGURE 3.1: Mean daily food consumption per week, calculated as mg of food consumed per g of body weight, for control (▨) and training (▨) groups. Mean body mass on the final training day of each week for control (●) and training (○) groups. Error bars denote standard error of the mean. At week 7, the training sessions were held twice each day (—+).

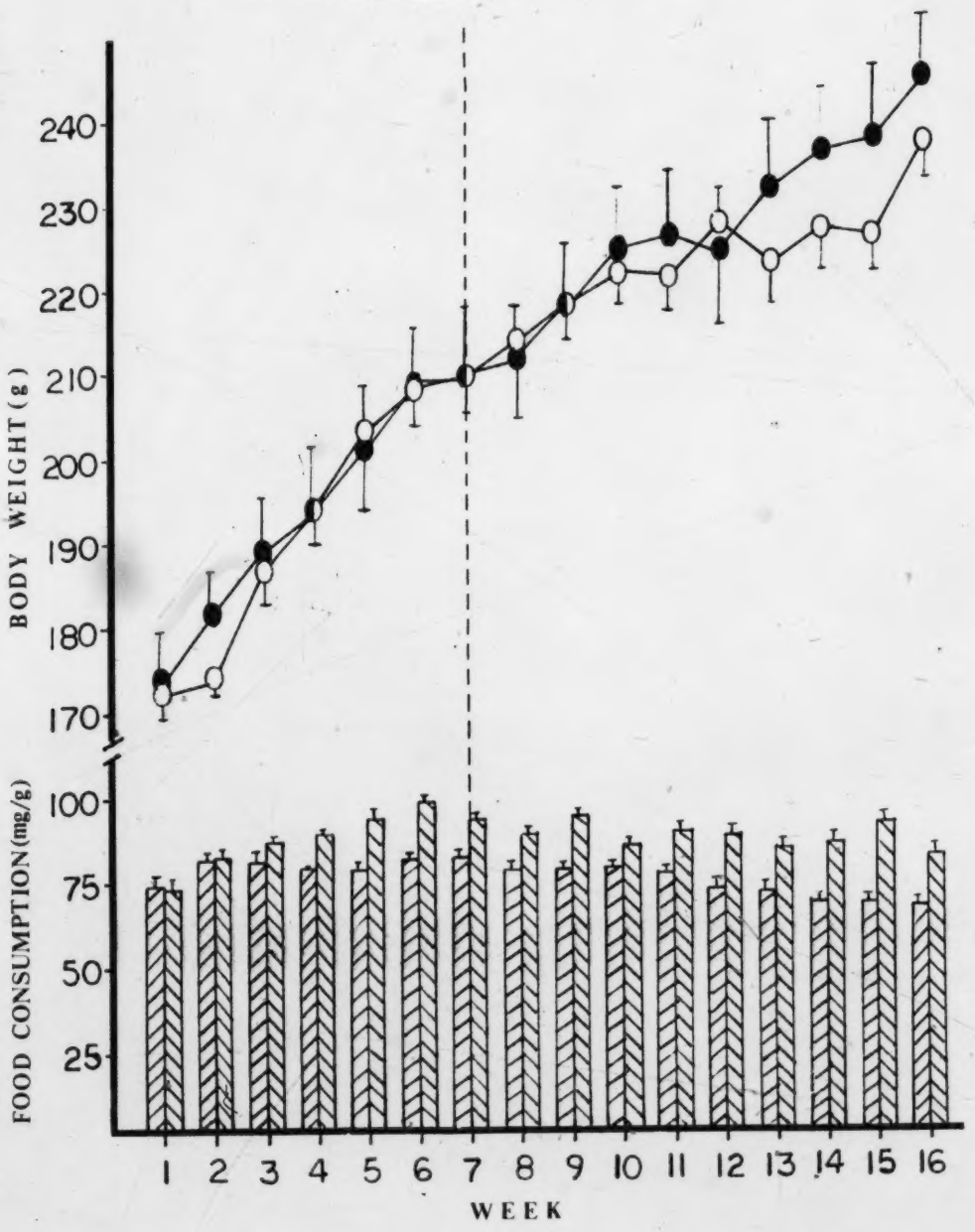

BODY WEIGHT (g)
240
230
220
210
200
190
180
170
FOOD CONSUMPTION (mg/g)
100
75
50
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
WEEK

TABLE 3.1: Summary of select anthropometic and protein concentration results of animals in the sedentary control and endurance training groups. Data are expressed as mean (standard error of the mean).

	CONTROL	TRAINED
Terminal Body Mass (g)	244.9 (8.1)	237.9 (2.8)
Heart Mass (mg)	900 (36)	1140 (58)
HM : BM ratio ($mg \cdot g^{-1}$)	3.7 (.15)	4.8 (1.8)
Muscle Mass (mg)		
Epitrochlearis	55 (3)	59 (2)
Tibialis Anterior	361 (15)	379 (7)
Normalized Muscle Mass ($mg \cdot g^{-1}$)		
Epitrochlearis	226 (10)	248 (5)
Tibialis Anterior	1471 (29)	1596 (23)
Total Muscle Protein ($mg \cdot g^{-1}$)		
Epitrochlearis	227.5 (4.8)	233.8 (4.0)
Tibialis Anterior	224.5 (3.7)	239.1 (4.0)

training program and vesicle type was not significant ($p>.05$).

Both the total (Mg^{2+} + Ca^{2+}) ATPase and Ca^{2+} ATPase activities in light and heavy vesicles isolated from animals of the training group were significantly depressed when compared to that of the controls ($p<.01$; Tables 3.5 and 3.6). In addition, the enzyme activities determined with light vesicles were significantly lower than those of heavy vesicles ($p<.01$). There was a significant interaction effect ($p<.01$) such that the heavy vesicles isolated from muscle obtained from trained animals had significantly depressed total and Ca^{2+} ATPase activities when compared to light and heavy vesicles isolated from control animals and light vesicles isolated from trained animals ($p<.01$). To assess the possibility of mitochondrial membrane contamination, the enzyme assays were repeated with the addition of 10 uM NaN_3. There was no significant difference observed in either type of vesicle in either experimental group demonstrating limited mitochondrial contamination (table 3.6).

Likewise, to assess the extent of the effect of Ca^{2+} efflux on the Ca^{2+} ATPase function, 15 uM ruthenium red (a calcium channel blocker) was added to the media. There was no difference in the Ca^{2+} ATPase activites of trials performed in the presence and absence of RR (Table 3.8).

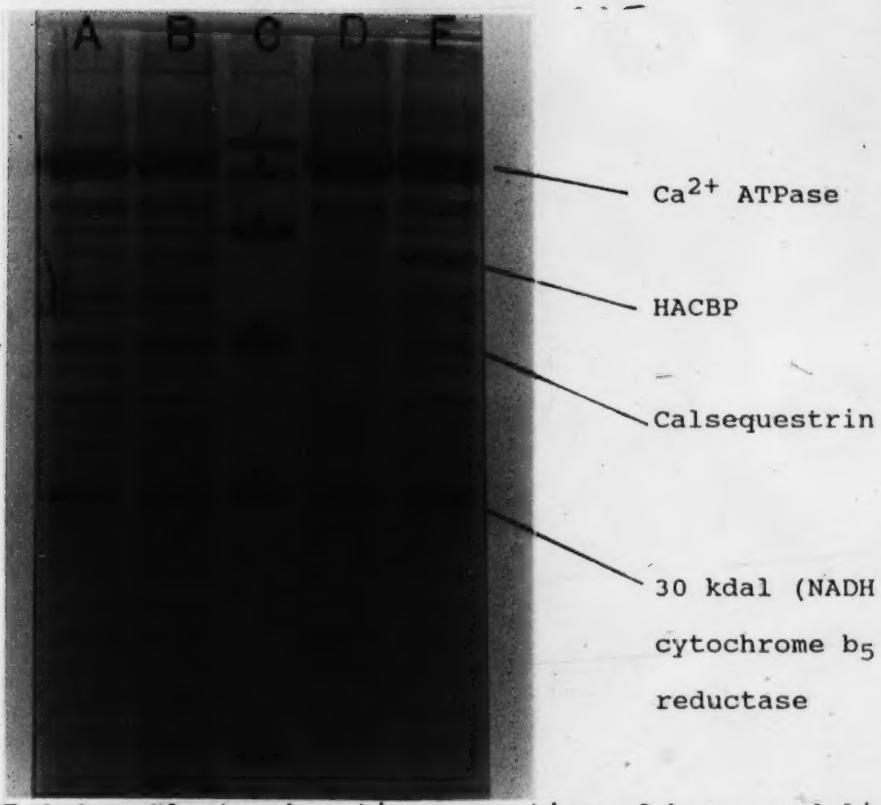


PLATE 3.1: Electrophoretic separation of heavy and light SR vesicles isolated from the superficial vastus lateralis muscles for rats in the Control and Training groups. The protein was denatured with SDS and layered onto polyacrylamide gels of 2% stacking and 10% running layers. The gels were stained with Coommasie Blue.

Column A: Heavy Vesicles from control animals

Column B: Heavy Vesicles from trained animals

Column C: Sigma Molecular Weight Standards (1 = 115 kdal, 2 = 94 kdal, 3 = 66 kdal, 4 = 45 kdal, 5 = 29 kdal)

Column D: Light Vesicles from control animals

Column E: Light Vesicles from trained animals

TABLE 3.2: Absolute Area (grams) and Relative Area (%) of select proteins as scanned by densitometry (LKB Ultroscan) at 540 nm. (Absolute values were obtained by weighing paper excised from under the densitometric peaks for a particular protein, relative areas were determined by expressing paper weight for a particular protein as a function of total paper weight).

	Light Vesicles Areas		Heavy Vesicles Areas	
	Relative	(Absolute)	Relative	(Absolute)
115,000 dalton, Ca^{2+} ATPase				
Control	667	(.792)	609	(.700)
Exercise	523	(.793)	518	(.558)
55,000 dalton, HACBP				
Control	38	(.036)	44	(.071)
Exercise	81	(.051)	92	(.097)
46,000 dalton, Calsequestrin				
Control	30	(.045)	62	(.051)
Exercise	37	(.112)	90	(.099)
30,000 dalton, NADH cytochrome b_5 reductase				
Control	107	(.127)	107	(.178)
Exercise	82	(.113)	229	(.247)

TABLE 3.3 Ratio of the 105 kdal and the 30 kdal proteins. (Since the same amount of protein was layered onto each gel column, the ratio was calculated with the absolute values).

	LIGHT VESICLES	HEAVY VESICLES
CONTROL	6.23	5.69
TRAINED	6.38	2.26

TABLE 3.4: Yield of SR protein ($ug \cdot g^{-1}$). Data are expressed as mean (standard error of the mean).

	LIGHT VESICLES	HEAVY VESICLES
CONTROL	756.8 (10.0)	826.0 (12.7)
TRAINED	739.3 (14.2)	758.5 (15.9)

Note. ANOVA Pooled training effect $p<.01$; ANOVA Pooled vesicle Type effect $p<.01$; post-hoc t-test revealed statistical difference only between the light and heavy vesicles of the control tissue ($p<.01$); N=4 in all cells

TABLE 3.5: Ca^{2+}, Mg^{2+} ATPase (total) activity (umol $Pi \cdot mg^{-1} \cdot min^{-1}$). Data are expressed as mean (standard error of the mean).

	LIGHT VESICLES	HEAVY VESICLES
CONTROL	3.81 (00.09)	3.37 (00.08)
TRAINED	3.66 (00.10)	2.15 (00.07)

Note. ANOVA Pooled training effect $p<.01$; ANOVA Pooled vesicle Type effect $p<.01$; signicant interaction effect, post-hoc ANOVA and t-test demonstrated that heavy vesicles from the training group were significantly different from those in any other block ($p<.05$); in addition, post-hoc t-tests revealed statistical differences between heavy vesicles from control and trained tissue ($p<.01$), between light and heavy vesicles from control tissue ($p<.01$) and between light and heavy vesicles from tissue obtained from trained animals ($p<.01$). There was no significant difference between light vesicles from trained and control animals. N=4 in all cells.

TABLE 3.6: Ca^{2+} ATPase activity (umol $Pi \cdot mg^{-1} \cdot min^{-1}$). Data are expressed as mean (standard error of the mean).

	LIGHT VESICLES	HEAVY VESICLES
CONTROL	3.73 (00.12)	3.27 (00.08)
TRAINED	3.55 (00.09)	2.05 (00.08)

Note. ANOVA Pooled training effect $p<.01$; ANOVA Pooled vesicle type $p<.01$; significant interaction effect: post-hoc AVOVA and t-test revealed that heavy vesicles from the trained group differed ($p<.05$) from all other blocks; in addition, post-hoc t-tests revealed statistical differences between heavy vesicles from control and trained tissue ($p<.01$), between light and heavy vesicles from control tissue ($p<.01$) and between light and heavy vesicles from tissue obtained from trained animals ($p<.01$). There was no significant difference between light vesicles isolated from trained and control animals. N=4 in all cells

TABLE 3.7: Ca^{2+}, Mg^{2+} ATPase activity (umol $Pi \cdot mg^{-1} \cdot min^{-1}$) with the addition of 10 uM NaN_3. Data are expressed as mean (standard error of the mean).

	LIGHT VESICLES	HEAVY VESICLES
CONTROL	3.86 (00.03)	3.38 (00.03)
TRAINED	3.62 (00.08)	1.99 (00.05)

Note. t-tests of enzyme activity with and without azide for each block resulted in no significant difference; N=4 in all cells for heavy vesicles; N=3 in cells for light vesicles.

TABLE 3.8: Ca^{2+}, Mg^{2+} ATPase activity (umol $Pi \cdot mg^{-1} \cdot min^{-1}$) with ~~the additon of~~ 15 uM ruthenium red. Data are expressed as mean (standard error of the mean).

	LIGHT VESICLES	HEAVY VESICLES
CONTROL	3.45 (00.09)	3.34 (00.05)
TRAINED	3.67 (00.04)	2.30 (00.05)

Note. t-tests of enzyme activity with and without RR resulted in no significant difference; N=4 in all cells

CHAPTER IV

Discussion

Applicability of Results

While citrate synthase was not assayed in the present investigation, Green et al., (1983) demonstrated pronounced increases in the activity of this enzyme, in superficial vastus lateralis. This illustrates that the presently employed training program was of sufficient intensity to recruit this type IIB fast glycolytic muscle, the muscle of prime interest in this study. This would suggest a fibre transformation at the metabolic level. Green et al., (1984) reported pronounced histochemical fibre transformations based on a staining procedure for myofibrillar ATPase. After their training program this group noted a decrease in type IIB fibres and an increase in type IIA and type I fibres in the plantaris muscle. Technical difficulty prevented Green et al., (1984) from reporting definitive results on the response of the superficial vastus lateralis from a histochemical viewpoint.

Body Mass and Food Consumption

The increased food consumption by animals in the training group is a typical response to exercise as demonstrated by Oscai et al., (1971), who observed increased intake to balance caloric expenditure in female rats. This group suggested that intake - output balance was a characteristic of light to moderate, long-term exercise and that in short

duration, severe exercise, appetite suppression may be observed. Katch et al., (1979) using male rats studied the relationship of exercise intensity upon appetite. They reported that animals which exercised for short, high-intensity bouts reduced their food intake to a greater degree than animals which were made to exercise for longer durations at lower intensity. Most notable however, was the observation that both groups consumed less food than the sedentary controls. Appetite suppression was not observed in the present investigation, despite the fact that the exercise was very intense.

In addition, the elevated intake failed to meet the needs of the caloric expenditure such that in the final quarter of the program there was a trend towards the rate of body weight gain of the training group failing to keep pace with that of the control group. It is also possible that the rats may have been prevented from meeting their caloric requirements. The rats (normally accustomed to eating at any time) were given less access to food in that by the 14th week they were spending less than 21 hours of each training day in their cages. In addition to this, one must consider elevation of temperature and the implication of minor injury upon appetite. Finally, the concentration of food energy in the chow must be considered. It is quite possible that the rats were not satiated, but that food bulk (low caloric density) linited caloric consumption.

Belcastro et al., (1980) proposed that lower body mass in trained animals may be an indicaton of lower body fat. Based on the observation that total and normalized muscle mass did not differ between sedentary control and endurance trained animals while there was a trend towards lower body weight of trained rats in the present study suggests that the hypotheses of decreased fat mass may be tenable in this case.

Green et al., (1983) reported statistical difference in body weight between groups in the final week such that the control group had greater mass. However, their population differed in strain, age, sex and initial body weight. Ahrens et al., (1972) pointed out a relationship between age and the adaptive capacity to biological stress in terms of decreased response of tissues to certain hormones. The mean age of the rats used in this project was 75 days (at training day 1) and while Green et al., (1983) did not report initial age, the fact that their rats were almost twice the size at any given time suggests that they were somewhat older. In addition, the rats in the Green et al., (1983) study were male. As outlined in the delimitations of this thesis, Oscai et al., (1971) proposed a sex linked interaction with the effect of exercise on appetite.

The fact that total muscle protein and muscle mass did not vary between groups illustrates that cellular dehydration was not a factor in body mass.

Heart size has been observed to correlate with oxygen

uptake, cardiac output and stroke volume (Astrand and Rodahl, 1977). In untrained animals the heart size is in constant proportion to body size across a population, but in endurance trained animals hearts are larger (Terjung, 1976). This change is usually manifested as an increase in ventricular mass (Longhurst et al., 1981) to a magnitude of 15% - 30% (Oscai et al., 1971). The magnitude of the exercise induced cardiac hypertrophy in the present investigation was 27%, which is in line with observations made by Grande and Taylor (1965), Longhurst et al., (1981) and Nutter et al., (1981). While physiologically significant, this difference was not statistically significant even when normalized for body weight.

Profile of the sarcoplasmic reticulum

a) SR isolated from control animals

The fractionation of SR into light and heavy vesicles produced samples which were comparable to published literature, in certain definable characteristics. Yield of SR was observed to be significantly greater in the heavy fraction of the membrane. This difference has not been reported previously and may be attributable to both the ultrastructural nature of the anastomosing light fraction and its relative lower concentration of both calcium binding proteins. The polypeptide patterns are in agreement with those of Louis et al., (1980) who demonstrated that the light fraction consisted of approximately 90% Ca^{2+} ATPase protein

while 70% of the protein of the heavy fraction was the Ca^{2+} ATPase. The lower proportion of this protein in the light fraction obtained in our case (Table 3.2) appears to be due to the extraordinary appearance of calsequestrin in the light vesicles. Examination of the absolute area of the densitometric scans of calsequestrin in control muscle showed very little difference in quantity of this protein between the two vesicle populations. This may well be a species difference as this data differs substantially from rabbit and dog SR literature.

Calsequestrin has been shown to occur in very limited quantities in the longitudinal SR from rabbit (MacLennan and Wong, 1971). However, the greater absolute quantities of both binding proteins calsequestrin and High Affinity Calcium Binding Protein (HACBP) in the fraction isolated from heavy SR are in agreement with the observation of Winegrad (1965) that at rest the majority of calcium is associated with heavy SR. In addition, our results demonstrate that almost 50% more 30 kdal protein is located in the heavy fraction. Wiehrer and Pette (1983) reported that in unstimulated type IIB skeletal muscle, the ratio of 105 kdal to 30 kdal proteins in unpurified SR was 14.1 +/- 3.5. In addition to the purity of their preparation, this team did not obtain subfractions of the membrane. The control tissue in the present investigation produced ratios of 6.23 and 3.93 for purified light and heavy fractions respectively, the intra

Table 4.1: Qualitative Summary of Results

Parameter	ES*	PT**	Present study	
			light	heavy
[Ca^{2+} ATPase]	↓	↓	↓	↓
[Calsequestrin]	↓	?	no change	↑
[HACBP]	↑	↑	↑	↑
[30 kdal]	↑	↑	↓	↑
ratio 115 / 30 kdal	↓	↓	↓	↓
SR yield			no change	↓
Ca^{2+} ATPase activity	↓		no change	↓

Legend: * Electrical Stimulation (see Table 1.4 for references)

** Physical Training (Green et al., 1984)

tissue variation being attibutable to both greater absolute yields of the ATPase and lower yields of the 30 kdal protein.

b) SR isolated from trained animals

Table 4.1 summarizes the results of the present investigation from a qualitative viewpoint. SR yield was altered by the training program in that a diminished quantity of the membrane protein was obtained in both light and heavy fractions in comparison to the control. Decreases in the relative proportions of the Ca^{2+} ATPase have been reported to occur after both electrical stimulation (Heilmann and Pette, 1979; Helmann et al., 1981; and Klug et al., 1983b) and high intensity training (Green et al., 1984). A remarkable finding of the present investigation was that the decrease in the relative proportion of this calcium translocating protein was limited to heavy vesicles. Likewise, the concentration of the 30 kdal protein has been observed to increase in SR isolated after electrical stimulation (Heilmann et al., 1981; Klug et al., 1983) and training (Green et al., 1984). Margreth et al., (1971) observed that prior to muscle differentiation, there was no difference between type I and II muscle fibres in terms of NADH-linked electron transport activity in the SR. (The fact that after differentiation, this system is more sophisticated in slow type muscle may suggest a role of motor activity). In the present study the 30 kdal protein almost doubled in heavy SR but was observed to decreased approximately 12% in the light fractions. HACBP

and calsequestrin underwent a significant training effect, increases being somewhat more pronounced in the heavy vesicle. The 115 kdal/30 kdal ratio which decreases after both electrical stimulation (Wiehrer and Pette, 1983) and training (Green et al., 1984) was limited to the heavy membrane subfraction after the present training regime.

A marked depression in Ca^{2+} ATPase activity was recorded after the training program (as noted by Klug et al., (1983b) after ES), however, the heavy membrane subfraction followed the morphological observation in that it showed much more of a depression. The fact that the heavy SR under went a decrease in both Ca^{2+} ATPase activity and in the fibre specific marker ratio of Ca^{2+} ATPase and 30 kdal proteins suggest that fibre transformations may have occurred as a result of the high intensity training program.

Heilmann and Pette, (1979) and Klug et al., (1983) showed decreases in Ca^{2+} activated ATPase activity of type IIB fibres which declined by more that 50% after continuous ES, to a level equal to or lower than that observed in type I fibres. The latter research group observed values for the fibre specific ratio of type IIA which approached that observed in type I skeletal muscle. Green et al., (1983) after carrying out the training program upon which the present study was based, observed values of the fibre specific ratio of type IIB fibres to resemble those recorded in control type IIA muscle. Complete transformation of type

II to type I fibres was specified by Green et al.,(1984) if and only if the concentration of the cytosolic calcium binding protein, parvalbumin, decreased to undetectable levels. Since this was observed to occur only in trained type IIA fibres, this group suggested that the long-term high-intensity training program induced a type IIB to IIA conversion and a type IIA to type I conversion. The fact that the results of the present program changed in the same direction and with at least the same magnitude confirms the suggestion that a training program is capable of inducing transformations of the functional characteristics of skeletal muscle.

Hikida et al., (1983), in an endurance training study with humans, demonstrated that strenuous training resulted in injury to skeletal muscle fibres. The question thus arises, as to the possibility og the appearance of regenerating and/or embryonic muscle fibres which may lead to misinterpretation of our results. However, Kuipers et al., (1983), after carrying out an endurance training program with rats, reported that necrosis in muscle fibres was restricted to segments of 150 - 1250 um. As discussed by Armstrong (1986), this fact is physiologically significant because the fibre can maintain its neural innervation and therefore its fibre classification.

Several hypothesis have been put forth to account for the transformations thought to occur after electrical stimulation

and training. The basic premise is that the continuous contractile activity leads to altered transcriptional activity. Helig et al., (1984) demonstrated a two-fold increase in total RNA following 8 days of continuous electrical stimulation while Klug et al., (1983a) observed the appearance of fibre specific mRNA after a similar protocol suggesting the activation of both transcriptional and translational activity. Several prominent trigger mechanisms have been proposed. Figure 4.1 summarizes the suggested cascade leading to decreased Ca^{2+} ATPase activity combined with current observations and speculation. Sreter et al., (1980) suggested that continuous contractile activity would create a situation where cytosolic calcium concentration was continually elevated. This situation would be further exacerbated by the observed decrease with time in the concentration of parvalbumin, the cytosolic Ca^{2+} binding protein. Klug et al., (1983a) have suggested that the elevated cytosolic calcium concentrations would signal the activation of calcium dependent proteases, while Martonosi et al., (1977) have proposed that synthesis of the Ca^{2+} ATPase could occur at a diminished rate. Any of these phemonena would lead to a depression in the concentration of the Ca^{2+} ATPase. However, training differs distinctly from electrical stimulation in that the bouts of contractile activity were interspersed with long recovery periods in a ratio of at least 1 to 6.

One novel observation of the present investigation was the increase in the membrane bound proteins calsequestrin and HACBP. Intuitively, these proteins would assist in the regulation and re-uptake of the sudden and persistent elevation of free calcium. An alternate mechanism may be working in the observed exercise-induced decrease in Ca^{2+} ATPase activity. Borchman et al., (1982) reported that SR membranes from type I muscle have 3 times the quantity of cholesterol than membranes of fast type muscle. During the fibre transformation process a change in the phospholipid matrix has been recorded after ES (Sarzala et al., 1982). As more cholesterol is incorporated into the membrane during the type II to type I transformation, and as more membrane bound proteins are synthesized and incorporated into the membrane, it is possible that membrane viscosity could undergo a marked increase. Cholesterol and integral membrane proteins both alter membrane fluidity by restricting the movement of hydrocarbon chains (Kimelber, 1977). This has an indirect effect upon the activity of membrane bound enzymes as a restricted hydrocarbon environment hinders conformational change. (A direct effect of cholesterol upon enzyme activity has been ruled out by Bennett et al., (1980), who showed that cholesterol is rigorously excluded from the phospholipids in an annular position about the SR Ca^{2+} ATPase). The loss of membrane fluidity could explain the exercise induced decrease in Ca^{2+} ATPase activity. It cannot be the sole factor,

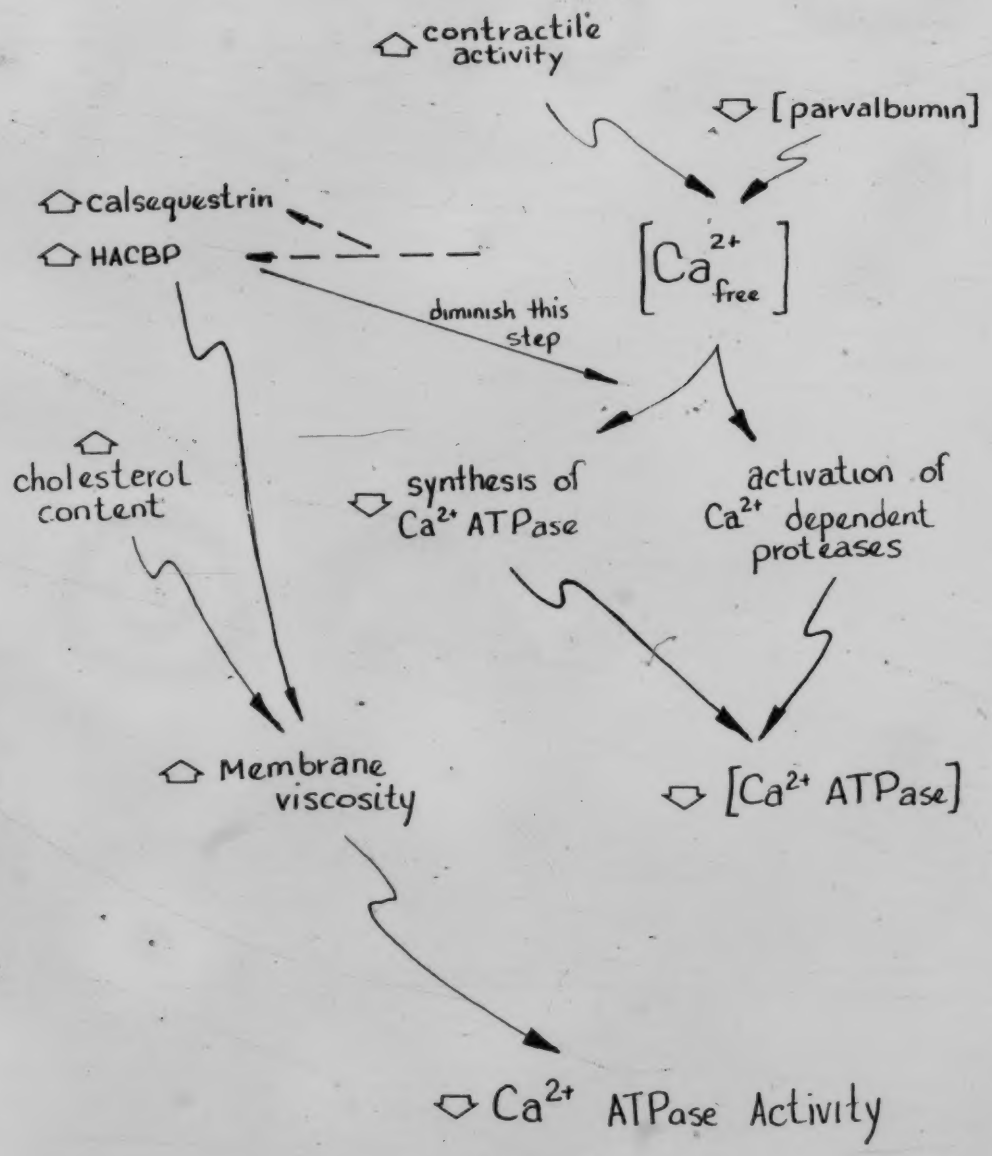


FIGURE 4.1: Mechanism of contractile-induced decrease in Ca^{2+} ATPase (see text). The dotted arrows represent novel data observed in this thesis project.

however, as Sarzala et al., (1982) demonstrated a time lag in the onset of changes in the lipid phase.

This explanation, however, does not account for the subfraction-specific alterations in the peptide profile and ATPase activity. Winegrad (1965) used labelled calcium to show that at rest, the majority of stored calcium resides in the terminal cisternae, but that early in the relaxation phase, calcium could be found in both regions of the membrane. The observation that exercise induced alterations of the SR were limited to the heavy portion presents two possible mechanisms. Firstly, the increase in the membrane bound calcium binding proteins occurs in the region where they are normally more abundant. Whatever mechanism triggers the cascade which eventually leads to decreased ATPase activity could also activate increased production and insertion of these proteins into the entire surface area. (Thus, the observed increase in calsequestrin and HACBP in both sub-fractions). However, the high concentration of these proteins in the terminal cisternae could invoke a localized increase in membrane viscosity ultimately leading to the observed decrease in ATPase activity.

Alternatively, the observed change in the peptide profile and ATPase activity may be dose- and time-dependent, such that a protocol involving electrical stimulation or physical training of longer duration may elicit comparable changes in the longitudingal SR.

CHAPTER V

Conclusions and Recommendations

Conclusions

1. Long-term high-intensity endurance exercise induces changes in the function of the SR of type IIB fibres such that they resemble type IIA fibres. However, total transformation of type IIB to type I fibres from a functional standpoint, may not be confirmed without observation of more of the fibre specific parameters (ie. parvalbumin). Never the less, based on the fact that this study achieved results in the same direction and of at least the same magnitude as Green et al., (1984) (who did demonstrate a histochemical, metabolic and limited contractile transformation with a similar protocol), confirms that fibre type transformation of type II to type I fibres is achievable with an exercise stimulus.

2. Long-term high-intensity training elicits fibre-specific changes (in yield of SR, peptide profile and Ca^{2+} ATPase activity) which are limited to Heavy (terminal cisternae) SR. The absence of change in Light (longitudinal) SR suggests that the SR consists of two phenotypically distinct elements.

Recommendations

1. To attempt to further clarify the proposed mechanisms of exercise-induced fibre type transformations, future work using this protocol should include:

a) a detailed profile of the phospholipid matrix.

b) analysis of the kinetics of the Ca^{2+} ATPase.

2. It is of interest to examine the extent of the effect of an exercise protocol upon the myosin heavy chains (ie methylhistidine residues) as previously demonstrated using an electrical stimulation protocol.

3. Any attempt to use this training program should allow for an attrition rate of up to 50%.

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APPENDIX A

Review of Literature

Sarcoplasmic Reticulum Structure and Function

The sarcoplasmic reticulum is a membraneous network which envelops the myofibrils of muscle cells. It performs an important role in excitation - contraction coupling as it acts as a Ca^{2+} storage reservoir as well as functioning to release and take up this cation.

The sarcotubular system consists of the SR (which itself consists of two components: the terminal cisternae and the longitudinal reticulum), and the transverse (t-) tubular system (Franzini-Armstrong, 1980). The SR and its associated structures are schematically depicted in Figure A.1. The terminal cisternae of the SR is in close association with the t-tubules while the longitudinal reticulum traverses the myofibrils between the terminal cisternae. One t-tubule and the two adjacent terminal cisternae constitute the triad (Franzini-Armstrong, 1980), and are connected to one another by membraneous extentions often referred to as "feet" (Franzini-Armstrong, 1980). These feet were observed to remain with the SR after isolation (Campbell et al., 1980).

Calcium release from SR is initiated by surface membrane depolarization, action potentials being propagated via t-tubules (Huxley, 1971). Calcium ions are released interactive

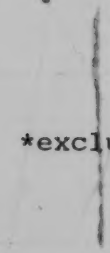

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Figure A.1: Schematic diagram of the SR and its associated structures. (Peachey, 1965). 40,000 X

relationship of actin and tropomyosin such that from the terminal cisternae (Winegrad, 1970; Miyamoto and Racker, 1982). Binding of calcium to troponin induces a conformational change in the myosin binding sites on actin become exposed, leading to myofibrillar contraction.

Calcium uptake occurs by virtue of the Ca^{2+} ATPase protein which can rapidly take-up cytoplasmic calcium, initiating myofibrillar relaxation (Meissner, 1975; Fabiato and Fabiato, 1979). Racker (1972) demonstrated that an enzyme, the Ca^{2+} ATPase, is responsible for transport by reconstituting purified ATPase in a phospholipid membrane and observing ATP-dependent Ca^{2+}-transport. Later, Hasselbach (1964) proposed a model whereby high affinity calcium binding sites on the cytoplasmic side of the membrane bind calcium. Upon coupling with ATP, the enzyme undergoes a conformational change where the binding site turns inward to face the luminal aspect. In association with the conformational change would be a decrease in the affinity of the binding site, resulting in the release of the calcium into the lumen of the S R. Two calcium ions are transported for each mole of ATP that is hydrolysed (Meissner, 1975). This reaction velocity is dependent upon the in vitro concentrations of Ca^{2+}, Mg^{2+}, and ATP as well as the temperature and the pH.

Freeze-fracture electron microscopy of the native membrane revealed an asymmetric structure (Deamer and Baskin, 1969). The cytoplasmic leaflet contained a high concentration of 9 nm

intramembraneous particles. In the membrane reconstituted after isolation of the Ca^{2+} ATPase, the intramembraneous particles were observed to be symmetrically distributed (MacLennan et al., 1971). However, Wang et al., (1979) showed an asymmetrical distribution when the phospholipid:protein ratio exceeded 88 moles per mole of ATPase. It was proposed that these particles represented the ATPase calcium-transport protein (MacLennan et al., 1971; Wang et al., 1979). Using electron microscopy on negatively stained native membranes, Stewart and MacLennan (1974) observed the cytoplasmic extensions of the ATPase as 4 nm particles. Martonosi (1975) demonstrated a good correlation between the appearance of the 4 nm particles and ATPase activity, in developing muscle. Scales and Inesi (1976) observed that the number of 4 nm particles exceeded that of the 9 nm particles in both native membrane and reconstituted vesicles. This represented the first suggestions of the possible oligomeric structure of the transport protein.

Phosphatidylcholine and phosphatidylethanolamine, the primary and essential phospholipids of the SR membrane (Knowles and Racker, 1975), were also observed to be asymmetrically distributed in the native membrane and in reconstituted vesicles with a lipid:protein molar ratio exceeding 88 (Wang et al., 1979). Phosphatidylethanolamine, along with the smaller quantities of phosphatidylserine, were observed to predominate in the cytoplasmic leaflet (Hidalgo

and Ikemoto, 1977; Sarzala and Michlak, 1978).

The calcium-dependent ATPase has been estimated to account for up to 70% of the total protein in the SR (MacLennan et al., 1971). Two extrinsic calcium binding proteins have been isolated: calsequestrin (MacLennan and Wong, 1971); and a high-affinity calcium binding protein (HACBP) (Ostwald and MacLennan, 1974). Campbell and MacLennan (1981) identified glycoproteins of the SR membrane. The 53000 dalton protein is a major intrinsic (membrane spaning) protein of skeletal muscle SR and is found in both the longitudinal and terminal cisternal components. Many researchers have reported the presence of a 30 kdal intrinsic membrane protein and a 34 kdal protein localized in the terminal cisternae (Campbell et al., 1980). While the latter protein was proposed to be part of the feet of the triad (Campbell et al., 1980), the former has not been assigned a functional role. Others (Margreth et al., 1971), have suggested that 30 kdal protein is NADH-cytochrome b_5-reductase. Finally, a proteolipid has been isolated from the membrane (MacLennan et al., 1972) but has not been assigned a functional role (MacLennan et al., 1972; Racker, 1972).

In the isolation and purification of SR vesicles, light and heavy fractions have been obtained (Meissner, 1975; Campbell et al., 1980). Analysis of these fractions revealed that the light fraction originated from the longitudinal component, the heavy fraction from the terminal cisternae. The primary

distinguishing factor was the presence of calsequestrin, found only in the heavy fraction (MacLennan and Wong, 1971). Campbell et al., (1980) demonstrated the presence of the feet in this fraction, suggesting that certain areas of the SR are specialized for particular functions.

a) Protein Composition of the SR

Since the calcium transporting ATPase protein will be discussed at great length throughout this review, this section will deal with the additional membrane proteins of the S R.

Calsequestrin is an extrinsic calcium binding protein found in the heavy SR fraction (Meissner, 1975; Campbell et al., 1980). MacLennan and Wong (1971), using SDS polyacrylamide gels, ascribed a molecular weight of 44,000 to this protein. However, since its electrophoretic mobility is dependent upon the conditions of pH and ionic concentration, Meissner et al., (1973), ascribed a molecular weight of 63,000 by using the Laemmli gel proceedure. Since over 1/3 of this protein is composed of glutamate and aspartyl residues, it is a highly acidic protein with a binding capacity of 1 umole Ca^{2+} / mg of protein and a dissociation constant of 800 uM in physiological media (MacLennan et al., 1971; Ostwald and MacLennan, 1974). MacLennan and Wong (1971) observed a conformational change in calsequestrin (including an increase in the helical content) upon binding calcium.

The high-affinity calcium binding protein was observed to be found in both the heavy and light SR (Michlak et al., 1980),

however, it was observed to be most highly concentrated in fractions originating from the t-tubules (Michlak et al., 1980). Within the S R, this calcium binding protein was observed to be present in a constant ratio with the Ca^{2+} ATPase (Michlak et al., 1980) and binds one mole of Ca^{2+} per mole of protein with a dissociation constant of 4 uM (Ostwald and MacLennan, 1974). However, while this protein has a high affinity for calcium, its concentration is small and its capacity for bound calcium is low (Ostwald and MacLennan, 1974). Michlak (1985) has proposed that the fact that it is present in relatively high concentrations in the t-tubules suggest a communicative function between the membranes of the t-system and the SR.

Using Concanavalin A binding, four glycoproteins of molecular weight 160,000, 63,000, 60,000 and 53,000 have been detected in the SR membrane (Michlak et al., 1980; Campbell and MacLennan, 1981). The 63 kdal protein was calsequestrin (Michlak, 1980; Campbell and MacLennan, 1981). The 53 kdal glycoprotein was observed to be in constant proportion with the ATPase in both the longitudinal and terminal cisternae membrane components, and only in very low concentrations in fractions isolated from the t-tubules (Michlak et al., 1980). The 53 kdal glycoprotein was not found to be in tight association with the ATPase (Campbell and MacLennan, 1981). This was observed to be a transmembrane protein since various fluorescence probes detected peptides on the cytoplasmic side

of the membrane (Michlak et al., 1980), while Endo H digestion of oligosaccaride chains occurred on the luminal side of the membrane (Campbell and MacLennan, 1981). The 53,000 dalton protein has not been ascribed a function although it has been suggested that, like the glycoprotein of the Na^+,K^+-ATPase, it may be responsible for transport of cations (Michlak, 1985).

An intrinsic proteolipid has been detected in SR membranes, but has not been ascribed a functional role. Racker (1972) suggested it might be a Ca^{2+} channel. While it is found in close association with the ATPase, purified vesicular fractions, free of the proteolipid, were observed to have full transport function (MacLennan et al., 1980).

Several researchers have reported the presence of an intrinsic membrane protein of molecular weight 39 kdal (Hidalgo and Ikemoto, 1977; Campbell and MacLennan, 1981). The latter group suggested that this protein was restricted to the heavy fraction of SR, however, Margreth et al., (1977) demonstrated that it was specific to the SR membrane of slow type skeletal muscle. Wiehrer and Pette (1983) proposed the use of 115 kdal/ 30 kdal ratio as a fibre-specific marker. Salviati et al., (1979) showed that this polypeptide was NADH-cytochrome b_5 reductase. They also detected the presence of a 16,700 dalton protein which co-electrophoresed with liver cytochrome b_5 (Salviati et al., 1981).

b) Cardiac SR Membranes

The sarcotubular system of cardiac muscle, like that of skeletal muscle, consists of sarcoplasmic reticulum and t-tubules. However, in the cardiac tissue of the mammal, Suzuki and Sugi (1982) observed a poorly developed t-system in atrial compared to ventricular fibres and a complete absence of t-tubules in isolated fibres of Purkinje. The cardiac analogue of skeletal SR terminal cisternae is referred to as interior junctional SR while that portion overlying the myofibrils is referred to as peripheral junctional SR (Michlak, 1985). Forty percent of the total protein of the cardiac muscle SR is the 100,000 dalton ATPase (Jones and Cala, 1981). Calsequestrin and the 53,000 dalton glycoprotein have been identified in cardiac SR, (Campbell et al., 1983), the former with a binding capacity of 300 umoles per mg of protein (Campbell et al., 1983). Several other low molecular weight proteins have been detected in the SR membrane isolated from cardiac muscle, the most significant being phospholamban. Louis et al., (1982), proposed that the protein is composed of three subunits: 11,000, 8,000 and 4,000 daltons, and that only the 11 kdal subunit could be phosphorylated in a calmodulin-dependent protein kinase system. Jones et al., (1979) observed that phosphorylation of phospholamban had a stimulatory effect upon Ca^{2+} ATPase activity and uptake. It was therefore proposed that phospholamban was tightly associated with the Ca^{2+} dependent ATPase in cardiac muscle S R (Katz, 1979).

c) Purification, Characterization and Reconstitution of the Ca^{2+} - dependent ATPase of Skeletal Muscle SR

The proceedure for purification of the Ca^{2+}-dependent ATPase involves solubilization with a non-ionic detergent followed by removal of the detergent by dialysis (Racker, 1972), sucrose gradient centrifugation (Warren and Metcalf, 1976) or affinity chromatography (Ikemoto et al., 1971). Several research groups (MacLennan et al., 1971; Meissner et al., 1973) investigated the amino acid composition of the Ca^{2+} ATPase. The observation that 43% of the residues were polar, substantiated the observation that a large part of the protein resided outside of the lipid phase. Tryptic hydrolysis has been utilized to cleave the enzyme and ascertain the fragments of primary importance in calcium function. The initial trypsin digest was reported to contain two fragments of 45 and 55 kdalton (Shamoo and MacLennan,1974; Shamoo, 1978). These fragments remained in association with the membrane and retained the ability to transport calcium. However, the transport function was lost upon further digestion of the 55 kdal fragment to 25 kdal and 30 kdal fragments. These researchers (Shamoo and MacLennan, 1974; Shamoo 1978) concluded that the site of phosphorylation could be found within the 39 kdal fragment while the 25 kdal fragment was responsible for Ca^{2+} translocation (Pick and Racker, 1979). Murphy (1977) reported that a particular lysine residue, referred to as the "essential lysine" was intimately involved

in phosphorylation in the 30 kdal fragment.

Several procedures, including labelling of particular residues with radioisotope reagents as well as cell-free synthesis, have been used to propose that the NH_2 terminus of the enzyme is located on the cytoplasmic side of the membrane (Reithmeier et al., 1980; Reithmeier and MacLennan, 1981). It was also predicted that the COOH terminus was located on the cytoplasmic side, due to the fact that during biosynthesis, it would be left on that side (Michlak, 1985). On the basis of sequenced and unsequenced portions of the protein, a folding pattern has been suggested such that the enzyme has a total of eight membrane crosssings (MacLennan and Reithmeier, 1982).

The isolated, solubilized enzyme has been reconstituted into a bilipid membrane system capable of sequestering Ca^{2+}. Early reconstitution studies by Racker (1972) involved a system which included the Ca^{2+} ATPase as well as phospholipids and proteolipid. Later, phosphatidylcholine and phosphatidylethanolamine were shown to be necessary to calcium transport (Knowles and Racker, 1975). The structural organization of the protein and lipid system appeared to be a necessary requirement as efficient calcium accumulating vesicles have been formed using synthetic phospholipids (Warren et al., 1974). Wang et al., (1979) concluded that a lipid:protein molar ratio greater than 88 was necessary to achieve enzyme activities comparable to the native membrane systen. Herbette et al., (1981) suggested that this may be a

function of the degree of penetration of the ATPase into the membrane bilayer in reconstituted vs native membranes. Studies examining enzyme activity and Ca^{2+} sequestration in reconstituted vesicles (MacLennan et al., 1980; Michlak et al., 1980) have revealed that the essential protein elements appear to be the Ca^{2+} dependent ATPase and the 53,000 dalton glycoprotein. The proteolipid did not appear to be an essential component.

d) Oligomeric aggregations of the Ca^{2+} ATPase

Various physical/chemical techniques have been used to determine the physiological aggregation state of the Ca^{2+} dependent ATPase in the native SR membrane. Freeze-fracture electron microscopy of the native membrane revealed that the intramembraneous particles numbered 5,000 to 6,000 per um^2 of membrane surface area (Scales and Inesi, 1976). However, Jilka et al., (1975) estimated that the number of discreet calcium transport enzymes in purified vesicles was 22,000 per um^2, leading Scales and Inesi (1976) to suggest that the microscopic particles which they had observed, represented 3-4 ATPase enzymes.

Cross-linking sulfhydryl reagents have also been used to investigate tha aggregation state of the Ca^{2+} ATPase in the SR membrane. Using Cu^{2+} -1,10 phenanthroline to stimulate sulfhydryl oxidation, Hebdon et al., (1979) observed the ATPase as a monomer, while others have observed dimeric

(Baskin and Hanna, 1979) and tetrameric (Murphy, 1976) aggregation. The lack of repeatability using cross-linking reagents leaves the question unanswered.

Detergent solubilization of the enzyme has been used to determine the smallest functioning aggregation state. Using non-ionic solubilization, LeMaire et al., (1976) observed valid enzymatic activities only in oligomeric formations. However, Moller et al., (1980) and Dean and Tanford (1978) have reported comparable ATPase activities in monomeric forms after attempting to optimize the in vitro conditions. Andersen et al., (1980) used fluorescence probes to observe covalent protein-protein relationships in both detergent solubilized and membrane bound enzymes. These data suggest that the ATPase may indeed exist in oligomeric form in the SR membrane.

e) The role of the lipid environment

The native SR membrane and thus that of reconstituted vesicles consists primarily of phosphatidylcholine and phosphatidylethanolamine and secondarily of phoshatidylserine and sphingomyelin (MacLennan et al., 1971). Removal of phospholipids inactivated the enzyme (LeMaire et al., 1976; Dean and Tanford, 1978), while readdition of the phospholipids reactivated the ATPase to its former level of activity (Martonosi et al., 1968). As noted in the previous section,

replacement of the phospholipids by detergent maintained the enzyme activtiy (Dean and Tanford, 1978). LeMaire et al., (1976), suggested that the key element may be the hydrophobic environment.

Cholesterol has been observed to be present in small quantities in the SR membrane (Drabikowski et al., 1972). Removal of this phospholipid did not have a measurable effect upon enzyme activity (Drabikowski et al., 1972) however, the introduction of additional cholesterol into the membrane resulted in decreased ATPase activity (Warren et al., 1975) possibly due to increased membrane viscosity. Warren et al., (1975), also noted that addition of cholesterol in a phospholipid depleted system affected enzyme activity, thought to illustrate a relationship between this phospholipid and the Ca^{2+} dependent ATPase. Notable is the observation by Borchman et al., (1982), that 3 times as much cholesterol (as well as a greater sphingomyelin : phosphatidylcholine ratio) existed in the SR membrane derived from slow type skeletal muscle. This group proposed that the lower enzyme activity observed in SR from slow-twitch muscle may be a function of membrane fluidity related to the phospholipid composition of slow SR membrane.

Warren et al., (1974) observed that the lipid annulus, the ring of phospholipids in immediate contact with the protein, must contain at least 30 molecules of lipid per ATPase molecule to support maximal enzyme activity. Hesketh et al., (1976) demonstrated that the physical properties of the lipids

of the annulus differed from that of the lipids of the non-annular membrane. This research team also observed that the physical properties of the annular phospholipids regulated enzyme activity. Bennett et al.,(1978) showed that the 30 molecules of phospholipid were distributed evenly between the luminal and cytoplasmic leaflets.

f) Kinetics of the Calcium-dependent ATPase

In the process of calcium uptake by the ATPase, energy from the hydrolysis of ATP is used to create a gradient of this ion across the membrane. In vitro systems have been observed to sequester calcium such that the cytosolic concentration decreases from 10^{-4} M to 10^{-7} M, the latter concentration being that observed in vivo, in muscle at rest (Hasselbach and Makinose,1962). Likewise, in the reverse process, the energy derived from the transmembrane gradient can be used by the ATPase to synthesize ATP from ADP and Pi (Makinose, 1972).

Two different ATPase activities have been observed to occur in the SR. A low rate, magnesium-stimulated activity (often referred to as the "basal" activity) does not require calcium for operation (Hasselbach and Makinose, 1962). The other ATPase activity requires both magnesium and calcium. For quantitative determination of the latter activity, one must subtract the "basal" activity from the total ATPase activity (Hasselbach and Makinose, 1962).

The initial event in Ca^{2+} translocation, is Ca^{2+} binding to

the enzyme. In investigations of the sequence of events, several research groups have reported that the rate constants in systems preincubated with calcium far exceeded those achieved in situations where ATP and Ca^{2+} were added simultaneously (Verjovski-Almeida et al., 1978; Inesi et al., 1980). This was thought to be due to the time required for the Ca^{2+} ions to saturate the binding sites.

The next detectable event is the phosphorylation of the enzyme. This was shown in early studies of the mechanism of calcium transport and was illustrated by the reverse mechanism whereby the formation of [^{14}C]-ATP occurred upon incubation of the enzyme with ATP and [^{14}C]-ATP (Hasselbach and Makinose, 1962).

Following enzyme phosphorylation, the calcium bound to the high affinity site becomes interalized. Cheisi and Inesi (1979) noted that this was demonstrated by a rapid burst of calcium uptake, manifested as a decreased amount of the cation which was extravesicular and available for chelation by EGTA. However, it was reported that this uptaken calcium was not immediately released into the lumen of the vesicle but rather remained occluded until certain enzyme catalytic steps had taken place (Dupont, 1980). A model for the active transport of Ca^{2+} (deMeis and Vianna, 1979), proposed the existence of high and low affinity calcium binding sites, an ATP-dependent change in affinity being central to calcium translocation. This model proposed that at the outset of the translocation

process, the binding site faces the cytoplasm and has such a high affinity for calcium that binding occurs even when the cytosolic concentration is below 1 uM. The enzyme would then undergo an ATP-induced conformational change which not only exposes the binding site to the lumen of the SR but also involves a decrease in the affinity of the enzyme for calcium. Calcium would thus be released into the lumen. A new cycle of transport could occur after hydrolysis of the phosphorylated intermediate, as the binding site undergoes the reverse conformational change, to face the cytoplasmic side and the affinity for calcium returns to the high state. This model has been supported by the investigations of Ikemoto (1975;1976) where a transmembrane calcium gradient was prevented by the use of "leaky" vesicles. In these studies, calcium was released from the ATPase upon addition of ATP but reassociated once hydrolysis of the enzyme had taken place. In summary, in the presence of calcium, the unphosphorylated enzyme exists in a high affinity state. An input of energy, such that achieved through ATP hydrolysis, is required to release the bound calcium via a shift in the phosphoenzyme affinity for Ca^{2+}. Calcium transport is inhibited when the intravesicular calcium concentration exceeds 10^{-3} M such that the low affinity binding sites (on the luminal surface) became saturated with Ca^{2+}. Figure A-2 illustrates the proposed reaction scheme of deMeis and Vianna (1979).

Cheisi and Inesi (1980) also note the liberation of one mole

of H^+ upon binding of one mole of Ca^{2+} to the high affinity binding site. Calcium - enzyme binding was observed to be affected by high H^+ concentration, implying Ca^{2+} : H^+ competition (Hill and Inesi, 1982). Later this group (Inesi and Hill, 1983) confirmed a competitive relationship by altering the concentration of H^+ at fixed Ca^{2+} and ATP concentrations. However, a reduced P_i production was also noted at high pH. This H^+ ion was not interpreted as having an inhibitory effect upon hydrolytic cleavage of the phosphoenzyme but rather as a necessary ion to replace the Ca^{2+} as it was released from the low affinity binding sites at the end of the translocation process.

The calcium dependent ATPase reaction cycle has been observed to be fully reversible. Makinose (1972) used calcium loaded vesicles in a medium, containing EGTA, inorganic phosphate and ADP to demonstrate calcium release via the reverse cycle. The reverse cycle begins with phosphorylation

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FIGURE A-2: Reaction steps in the ATP-dependent translocation of calcium by the Ca^{2+} ATPase of the SR. (deMeis and Vianna, 1979).

1 + 2 : Formation of the $(Ca^{2+})_2$ - ATP - enzyme complex

3 : Phosphorylation of the enzyme and release of ADP

4 : Formation of phosphorylated enzyme intermediates with decreasing affinity for Ca^{2+}

5 : release of Ca^{2+} into the lumen of the SR

6 : hydrolysis of the phophorylated intermediate

7 : release of inorganic phosphate on the cytoplasmic side of the membrane

of the ATPase by Pi, followed by calcium efflux and phosphoryl transfer to ADP (Barlogie et al., 1971; Makinose, 1972).

In the forward cycle, ATP provides the energy required for the formation of the acyl phosphate. In the reverse cycle, energy is derived from the transmembrane calcium phosphorylation in the absence of a transmembrane gradient. gradient (Makinose, 1972). However, both leaky vesicles (deMeis and Masuda, 1974) and purified ATPase (Knowles and Racker, 1975; Hasselbach, 1978) have been used to demonstrate the reverse reaction. Observations made in the absence of this gradient, were that the inorganic phosphate did not react with ADP to form ATP (Knowles and Racker, 1975; deMeis, 1976), however it was also shown (Knowles and Racker, 1975) that the basic requirememt for phosphorylation of the nucleotide was saturation of the low affinity calcium binding sites.

g) Regulators of enzyme activity: $[Ca^{2+}]$, [ATP] and pH

The rates of calcium transport and ATP hydrolysis have been shown to depend upon calcium concentation (Hasselbach, 1978; Ikemoto, 1982). The rate peaks within seconds of ATP addition (Inesi, 1981), but declines thereafter, due to inhibition by the increasing intravesicular calcium concentrations (Inesi, 1982). Techniques used to observe initial and maximal activity rates include stopped flow mixing and chromogenic ion indicators (Inesi and Scarpa, 1972). Alternatively, Verjovski-Almeida et al., (1978) used H+ indicators which

detected post-hydrolysis production.

Studies on the effect of calcium concentration have necessitated the use of EGTA to bind and inactivate extravesicular calcium. The EGTA then acts as a buffer system when $CaCl_2$ is added to the medium to activate the enzyme (Fabiato and Fabiato, 1979).

The sigmoidal dependence of the ATPase activity upon calcium concentration (Vianna, 1975; Neet and Green, 1977) and the 2 : 1 molar ratio of Ca^{2+} transport and ATP hydrolysis suggest cooperative binding. Classes of binding sites have been indirectly observed (Fiehn and Migala, 1971; Ikemoto, 1975); a high affinity site (Kd < 1 uM, Meissner et. al., 1975) was suggested to exist on the cytoplasmic surface of the ATPase (Ikemoto, 1975; Inesi et al., 1980). The ATPase must bind calcium prior to ATP hydrolysis. Various techniques such as ESR spectroscopy (Coan and Inesi, 1977), kinetics of sulfhydryl reactivity (Ikemoto et al., 1978) and determination of intrinsic fluorescence (Dupont and Leigh, 1978; Ikemoto et al., 1978) have been utilized in studies which proposed that a conformational change in the enzyme occurred upon binding calcium. Inesi et. al., (1980) suggested that this change occurred after binding of the first Ca^{2+} a characteristic of cooperativity.

The Ca^{2+} ATPase reaction is also dependent upon the concentration of ATP such that the minimum required for activity has been observed to be 0.5 - 50.0 uM, the enzyme

activity increasing with increasing concentrations of ATP (Yamamoto and Tonomura, 1967; deMeis and deMello, 1973; Vianna, 1975). It has been suggested that the relationship between ATP concentration and enzyme activity is not a simple one. Verjovski-Almeida et al., (1978) observed that the initial phosphorylation of the enzyme by ATP was limited to the lower concentration range. Later, it was noted that the hydrolysis of the phosphoenzyme was activated by high ATP concentrations (Verjovski-Almeida and Inesi, 1979).

Lastly, pH is a regulator of ATPase activity. Specifically, pH influences the interaction of the ATPase and inorganic phosphate (Beil et al., 1977), thought to be due to Pi ionization. Since pH was observed to have no effect in the presenced of organic solvents, (deMeis et al., 1980), it was proposed that pH plays a role in ionization of protein residues.

The pH was also observed to play a role in the mechanism of calcium binding to high affinity sites. At high pH, the calcium affinity increases, while at low pH, the affinity and the extent of cooperativity increased, assumming that Ca^{2+} and H^{+} compete for interacting sites on the enzyme (Hill and Inesi, 1982).

Likewise, pH was observed to have an effect upon calcium binding to low affinity sites (Verjovski-Almeida and deMeis, 1977) as well as the calcium concentration effect of ATP formation. DeMeis and Tume (1977) in studying the reverse

reaction, reported that the pH had an effect upon the calcium concentration dependence of ATP formation. In their experiments, they fixed the pH at 5.0 and added only enough calcium to saturate the low affinity sites. They observed that if ADP were added to the solution, and the pH was rapidly adjusted to 8.0, ATP would be formed. They interpreted this decreased need for Ca^{2+} to be a result of decreased competition with H^{+} ions.

Ultrastructural and Biochemical Differerences between Fibre Types

Stereological analysis, which involves compiling two-dimensional electron micrographs for a three-dimensional ultrastructural view, has been used to create fibre-specific profiles of skeletal muscle. Mitochondria, patterns of capillarization, S R structure, lipid deposition as well as myofibril, Z-line and the T-system dimensions have been studied. Table A.1 compares stereological findings of types I and IIB fibres (Eisenberg et al., 1974; Eisenberg and Kuda, 1975).

Z-discs or Z-lines, the anchoring structure for actin filaments from adjacent sarcomeres, are considered to be the best ultrastructural parameter for fibre-type classification, because the Z-line is characteristic for a given fibre. The width is greatest in type I fibres (Tomanek et al., 1973) and narrower and less dense in type II fibres (Gauthier, 1974;

TABLE A-1: Summary of selected ultrastructural differences between fibre types (longitudinally sectioned rat skeletal muscle).

PARAMETER	TYPE I (SOLEUS)	TYPE II (VASTUS LATERALIS)
	Eisenberg et al., (1974)	Eisenberg and Kuda, (1975)
Blood Vessels	2.07 +/- 0.37%	0.8 +/- 0.3%
Fibre Diameter	33.0 +/- 2.2um	43.2 +/- 2.6um
Sarcolemmal Surface Area	11.6 +/- 1.3$um^2/100um^3$	7.2 +/- 0.6$um^2/100um^3$
Sarcomere Length	3.4 +/- 0.2um	2.95 +/- 0.1um
A-Band Volume	52.6 +/- 2.9$um^3/100um^3$	59.4 +/- 2 $um^3/100um^3$
I-Band Volume	47.8 +/- 2.5$um^3/100um^3$	40.6 +/-1.9$um^3/100um^3$
Myofibril Diameter	1.06 +/- 0.08um	1.12 +/- 0.07um
Z-Line Width	1420 +/- 30 A	610 +/- 10 A
Mitochondrial Volume Density	4.85 +/- 0.66%	1.91 +/- 0.18%
T-system Density	0.14 +/- 0.01%	0.27 +/- 0.02%
SR Volume	2.23 +/- 0.21%	4.59 +/- 0.29%

Eisenberg and Kuda, 1975). However, there exists considerable overlap of Z-line widths within A and B subtypes of type II fibres (Eisenberg, 1983).

Eisenberg (1983) reported that type I fibres exhibited five prominent M-lines while type IIB exhibited only three prominent M-lines by stereological analysis. The intermediate, IIA fibres exhibited three prominent M-lines bordered by two weak lines.

The T-tubules, the central element of the sarcotubular triad, was observed to be elliptical in cross-section (Eisenberg et al., 1974). Eisenberg and Kuda (1976) observed that the T-system of the type II fibres was two-fold more extensive than that of the type I fibres. Indeed, in type I fibres, T-tubules were often observed to be flanked only along one side by junctional cisternae, such that diads rather than triads were formed (Schiaffino et al., 1970).

Eisenberg et al., (1974), and Eisenberg and Kuda (1975), viewed the location of the cell nuclei in different fibre types. They reported that, in type I fibres, the nuclei were elongated ellipsoids found in the cell periphery while those in type II fibres were small, sparse and located close to the sarcolemma.

Like the proteins of the S R, myosin from different types of fibres differ in the protein electrophoretic pattern. Type I fibres differ from type II fibres by virtue of specific alkali and DTNB myosin light chains as well as heavy chains

(Sreter et al., 1973). Heavy chains form type II fibres are exclusive in their possession of 3-methylhistidine (Margreth et al., 1974a). Types IIA and IIB may be distinguished from one another immunohistochemically, base on the fact that they contain different heavy chains (Mabuchi et al., 1982) but identical light chains. Lastly, the myosin ATPase activity of type II fibres is greater than that of type I fibres (Margreth et al., 1972), a fact traditionally relied upon in histochemical detection techniques.

Electron microscopy has revealed that capillarization is greater around highly oxidative fibres than low oxidative fibres (Hoppeler et al., 1981). Likewise, Eisenberg and Kuda,(1976) observed that capillary supply and therefore oxygen supply to type IIB fibres was sparse. These characteristics are influential upon the enzyme profile, such that respiratory chain enzyme activity (ie. cytochrome oxidase and succinic dehydrogenase activity) are much higher in type IIA than type IIB fibres (Margreth et al., 1972; 1974a). It seems intuitive then, that glycogen particles were observed more prominently in type IIB fibres (Tomanek et al., 1973) in guinea pig skeletal muscle. (However, Schmalbruch (1979) observed that in human skeletal muscle, glycogen particles were found only in type I fibres). Tomanek et al., (1973) also reported a preponderance of lipid inclusions in type I and type IIA fibres, a characteristic rarely observed in type IIB fibres. In assocation with an increased reliance upon lipid

as an energy source in endurance activity, Holloszy et al., (1977) observed a tendency for trained athletes to have higher intracellular lipid volumes.

Several research groups have noted a wide variation in content of mitochondria within type II fibres (Schiaffino et al., 1970; Margreth et al. 1974a) and have suggested that the variable development of the S R between all fibre types was the only ultrastructural feature which bore a direct relationship to the speed of contraction. The mitochondrial content appeared to be related to resistance to fatique (Schiaffino et al., 1970).

Despite the fact that the mitochondrial content is readily altered by increased demands of the muscle, others consider it a prominent differential feature betweeen fibre types. In the type I fibres, mitochondria are found predominently in the peripheral annulus oriented transversely at the level of the I-band (Eisenberg et al., 1974). More than half are grouped as pairs (Ogata and Yamasaki, 1985). Like type I fibres, the mitochondria of the type IIB fibres are located predominantly in the outer annulus in longitudinal chains, however, in the core of the fibre, type IIB mitochondria are most frequently grouped singly and oriented on the side of the Z-line (Eisenberg et al., 1974). Type IIA fibres may be distinguished from the other types by a moderate number of mostly thin-type mitochondrial columns (Ogata and Yamasaki, 1985).

From a biochemical viewpoint, mitochondrial calcium uptake in type I fibres exceeds that in type II fibres by as much as 33% (Sembrowich et al., 1985). Margreth et al., (1974a), using electron microscopy demonstrated a more extensive development of the inner membrane infoldings of the mitochondria to type II fibres, a fact presented to account for increased activity of glycerol-3-phosphate dehydrogenase, an inner mitochondrial menbrane enzyme.

Stereologically, the SR was observed to consist of two components: the terminal cisternae (which runs adjacent to the T-system and contains grannular material) and the simple longitudinal tubules (which connect adjacent terminal cisternae and have clear lumen) (Eisenberg et al., 1974). The type I fibres were observed to have frequent branches and fenestrations between the longitudinal tubules (Eisenberg et al., 1974). Morphologically, the reconstituted vesicles of fragmented SR of type IIA and IIB fibres had a grannular appearance with projecting knobs (30 A) on the outer surface (Margreth et al., 1974a). These failed to become loaded with calcium oxalate (an ion-chelator complex capable of diffusing through the membrane) after in vitro incubation (Margreth et al., 1974a). Wang et al., (1979) observed the following ratio of active site (ie. transport protein) density: IIB = IIA > I.

Sembrowich et al., (1985) noted a greater yield of SR from type II fibres than from type I fibres, some researchers

reporting up to a two-fold difference (Feihn and Peter, 1971; Wang et al., 1979). Electrophoretic patterns from different fibre types revealed fibre-specific differences only in the number and mobility of minor protein conponents, of lower molecular weight (Margreth et al., 1974b). This group reported a reduced amount of the approximately 100,000 dalton protein in type I fibres when compared with type II fibres. This difference was estimated to be 1/5 of that observed in type II fibres (Margreth et al., 1974b). It has been documented that the highest concentration of the Ca^{2+} ATPase and calsequestrin is found fibres of type IIB (Zubrzycka-Gaarn et al., 1982; Wang et al., 1979), and that the ratio of the concentration of the 30 kdal protein is: type I > type IIA > type IIB.

The physiological manifestations of this discrepancy in the yield of SR and the concentration of the primary calcium transport protein, were noted by Margreth et al., (1974b). They observed that, in type I fibres, the SR appeared to play a negligable role in calcium movements linked to relaxation. The initial rate of Ca^{2+} accumulation in vesicles prepared from type IIA fibres was as much as 10 times that recorded in type I fibres. This has been a consistent observation, as others have reported that the maximum rate of calcium uptake was at least 4 times higher in type IIB than type I fibres (Feihn and Peter, 1971, Kim et al., 1981). This fibre specific discrepantcy in transport rates appeared to be more

than just a function of enzyme concentration, as Wang et al., (1979) observed variation between fibre types with reference to the affinity of the Ca^{2+} ATPase for calcium.

Since the formation of the phosphorylated Ca^{2+} ATPase enzyme ($E^{\sim}P$ coupling) is representative of the calcium translocation, the rate of phosphorylation and the rate of $E^{\sim}P$ decomposition is characteristic of the S R as a calcium pump (Wang et al., 1979). The rate of phosphorylation is greater in skeletal than cardiac muscle (Sumida et al., 1978 in Wang et al., 1979). Wang and Schwartz (1981) and Wang et al., (1979) observed that the half time to phosphorylation of the enzyme was shorter and that the rate of $E^{\sim}P$ decomposition was higher (by as much as 4 times, Zubrzycka-Gaarn et al., 1982), in type IIB fibres in comparison to type I fibres. The rates of these two reaction steps correlated well with the isometric half relaxation times; relaxation time being six-fold higher in type IIB (Eisenberg and Kuda, 1975). Wang and Schwartz (1981) suggested that type I fibres have a monophasic decomposition, while type II fibres decompose in a biphasic manner.

Exercise Training and Skeletal Muscle Fibre Composition in Humans

Most research employing training programs have illustrated the local adaptability of muscle to the stress of exercise. These adaptations have been manifested as increases in maximum oxygen consumption (Gollnick et al., 1973; Saltin et al., 1976; Orlander et. al, 1977; and Andersen and Henriksson, 1977) and by increased activity of enzymes involved in aerobic metabolism (Gollnick et al., 1973; Thorstenson et al., 1974; Saltin et al., 1976; Orlander et al., 1977; and Green et al., 1979).

Many human training programs have endevoured to elicit fibre interconversion. Gollnick et al., (1973), conducted an intense cycling program which failed to demonstrate a change in the percentages of type I slow twitch and type II fast-twitch fibres. Saltin et al., (1976) demonstrated the same absence of change in an intense one-legged endurance and sprint training program. However, it was noted that muscle hypertrophy occurred which was specific to the training program. Endurance training lead to hypertrophy of type I fibres while sprint training caused hypertrophy of both type I and type II fibre types, underlining the importance of exercise intensity in terms of recruitment.

While conversion of the major fibre types has not been observed in human studies, transitions within type II sub-

types have been documented. Green et al., (1979), in a longitudinal examination of trained Hockey players pre- and post- season, observed an increase in the percentage of type IIA fibres after the latter time period. Andersen and Henriksson (1977) conducted an intense cycle program in males and observed an increase in the percentage of type IIA fibres when expressed as a percentage of total type II fibres. These studies suggest a IIB to IIA conversion in response to endurance training. Jansson et al., (1978) conducted aerobic and anaerobic endurance training programs for a group of male long-distance runners. After anaerobic training, the subjects had a lower percentage of type I fibres and a higher percentage of type IIC fibres than that observed after aerobic training. In addition, these subjects showed an increase in types IIA and IIB fibres and an increase in the ratio of B:A. This group of researchers interpreted the results as the hypothesis that type IIC fibres were fibres in a transitional stage between a type I to type II conversion, such that anaerobic training ellicts a type I to type IIC conversion while aerobic training ellicits a type IIC to type I transition. This was the first such data which had suggested that any sort of interconversion of the major fibre types could occur through an exercise stimulus.

The major drawback of the studies done to date with humans involved in the physical training process appears to be a technical one. All fibre classification had been done on the

basis of histochemical staining proceedures and therefore relied upon the metabolic state of the samples. It may be concluded therefore, that with the exception of metabolic adaptation these training studies have failed to demonstrate that the exercise stimulus can alter fibre composition in humans.

Electrical Stimulation and Skeletal Muscle Fibre Composition in Animals

Much of the research which has examined the mechanism of muscle plasticity has make use of electrical stimulation or motor nerve cross re-innervation. Transformations of fibre type were thus elicited by changing the frequency of nervous impulse and thereby the pattern of contractile activity.

a) Metabolic Changes in Skeletal Muscle with Electrical Stimulation

Electrical stimulation protocols were observed to induce changes in the metabolic profile such that there was a decrease in enzymes of anaerobic metabolism and an increase in key enzymes of the citric acid cycle, beta-oxidation of fatty acids and ketone body utilization. This transformation lead to a complete loss of the histochemically observed mosaic-like pattern of "white, red and intermediate" fibres (Pette et al.,1972; Hudlicka and Tyler, 1984; and Buchegger et al.,1984). This shift towards aerobic metabolic metabolism means that there is a greater resistance to fatigue in stimulated skeletal muscle, therefore the fast type muscle, considered to be metabolically transformed to slow twitch muscle actually exceeds the native slow muscle in this parameter. Some researchers have therefore referred to the transformed muscle as "super red."

Reichmann et al., (1985) observed a 40-60% decrease in the

activity of extramitochondrial enzymes. With the exception of hexokinase, all studies employing continuous low frequency stimulation protocols have shown significant decreases in the extramitochondrial enzymes of glycolysis. Decreases have been observed in the activity of pyruvate kinase, phosphofructokinase tricsephosphate dehydrogenase and lactate dehydrogenase (references as summarized in table 1.3). Both Pette et al., (1973) and Harris et al., (1982) observed LDH isozyme shifts from M-type (LDH-5) to H-type (LDH1 as well as small quantities of the hybrid isozymes LDH 2,4,4). Hexokinase activity rose as a result of the continuous low frequency stimulation protocol,as early as 2 days after the onset of the program (Reichmann et al., 1985) by factors of 4-fold (Hudlicka et al., 1984) to 8-fold (Helig and Pette, 1980). Extramitochondrial enzymes for gluconeogenesis and glycogenolysis, fructose-1,6-diphosphatase and glycogen phosphorylase respectively, decreased in response to electrical stimulation. Pette et al., (1973) also observed the decline in the activity of anaerobic enzymes, creatine kinase and adenylate kinase.

Stimulation induced increases in the key enzymes of the citric acid cycle have been documented by many researchers. Increased activity of succinic dehydrogenase have been noted, Pette and Tyler (1983) and Reichmann et al., (1985) making use of microphotometric techniques for determination of enzyme activity within individual fibres. Citrate Synthase activity

has been observed to increase 2-3 fold in response to the stimulation (Pette et al., 1972,1973; Harris et al., 1982; Buchegger et al., 1984; Hudlicka et al., 1984; Reichmann et al., 1985). Fatty acid oxidation is facilitated in stimulated muscle by virtue of increases in 3-hydroxy-CoA-dehydrogenase (Pette et al., 1973 and Hudlicka et al., 1984 recording increases in the order of 2-3 fold) and Palmitoyl-CoA-dehydrogenase. Hudlicka et al., (1984) recorded a 60 % increase in the latter enzyme. However, Reichmann et al.,(1985) note that stimulation did not lead to an increase in the concentration of circulating ketones concluding, therefore, that the observed increase in ketone body oxidation in stimulated tissue was induced by the increased contractile activity.

(b) Stimulation-Induced Alterations in the Function of the SR

Functional changes, manifested as alterations in the structure and function of the SR have included changes in the initial and maximal rates of Ca^{2+} uptake, SDS electrophoretic peptide pattern and the concentration and/or ratio of key marker membrane and cytosolic proteins.

Studies using techniques for the isolation of fractionated SR membrane vesicles have generated much data on stimulation-induced functional changes. These changes are summarized in table 1.4. Kinetic studies by Heilmann and Pette (1979),of

the vesicles have shown decreases in Ca^{2+} activated ATPase activity and initial and total rates of calcium uptake.

Parvalbumin, a major calcium binding protein in fast-type skeletal muscle was consistently observed to decrease towards levels found in slow type muscle in response to stimulation. As outlined by Klug et al., (1983a), if the concentration of parvalbumin decreases, there is an increase in the level of free calcium in the cytoplasm. This phenomenon has been observed to be followed by a decrease in the synthesis of Ca^{2+} ATPase. Martonosi et al., (1977 in Heilmann and Pette, 1979) suggest that in embryonic muscle cell, a decrease in intracellular calcium is the primary stimulus for the synthesis of the Ca^{2+} ATPase. Increased cytoplasmic calcium would lead to a massive influx into the SR (and possibly the mitochondria (Pette et al., 1985b)). In dystrophic muscle, increased intravesicular calcium from increased influx has been observed to activate Ca^{2+} -dependent proteases (Pette et al., 1985b). Kameyama and Etlinger (1979) confirmed this with calcium ionophores. Thus it appears that changes in the concentration of parvalbumin may be linked to the stimulation-induced changes in ATPase concentration and function.

The 7-9 nm particles observed (by electron microscopy) on the concave face of the FSR vesicles and thought to be the structural equivalent of the Ca^{2+} transport ATPase (MacLennan et. al., 1971 in Heilmann et al., 1981), are significantly lower (particles/unit area) in slow muscle than fast muscle

(Bray and Rayns, 1976 in Heilmann et al., 1981). Heilmann et al., (1981) using freeze fracture electron microscopy, observed a 75% decrease in the amount of such particles located on the concave (A) face of vesicles isolated from stimulated muscle. Further, they observed an increase in the number of particles located on the convex (B) face. Therefore, the ratio of intramembraneous particles between A and B faces is reduced by stimulation. These researchers suggest that the reduced asymmetry of the membrane may be the result of an altered protein/lipid ratio and a rearrangement of the phospholipid pattern. Sarzala et. al., (1982) reported that chronic stimulation did not change the total lipoid/protein ratio but did cause a decrease in the phospholipid pattern. This was manifested as a 50 - 75% decrease in phosphatidyl choline content and an increase in lysophosphatides and non-esterified fatty acids in the SR membrane. They proposed that this new stimulation-induced membrane composition lead to an increase in membrane fluidity, a fact put forth to account for the change in membrane assymetry.

The ratio of 115 kdal/ 30 kdal membrane protein, suggested to be a highly sensitive marker of fibre-type specific differences in the SR, varies significantly between the three fibre types (Wiehrer and Pette, 1983). They report ratios of 14.1: 3.8: 1.2 for types IIB, IIA and I respectively. Early changes in this parameter were observed to occur within 2 days

of stimulation and reach values equivalent to native slow muscle within 3 weeks of chronic stimulation (Wiehrer and Pette, 1983).

c) Contractile Changes in Skeletal Muscle with Stimulation

Sensitive immunohistochemical and biochemical techniques of more recent investigations have allowed the elucidation of fibre-specific differences and therfore alterations in the contractile elements. Early physiological studies observed stimulation induced decreases in time to peak tension and increases in time to 1/2 relaxation and twitch tension ratio. More recent work in this area has concentrated on molecular changes in the contractile machinery during stimulation induced fibre-type transformations. Decreases in Ca^{2+} activated myosin ATPase activity have been manifested by alterations in the light chain and heavy chain components of the myosin molecule. Many authors (Sreter et al., 1974;1975; Hudlicka et al., 1982b; Sreter et al., 1982; and Buchegger et al., 1984) have reported the decrease in the fast myosin light chains in stimulated muscle. In particular, the DTNB (phosphorylatable) chains decreased before the alkali light chains. Seedorf et al., (1983) and Brown et al., (1983) observed that the decrease in light chains 1f and 2f and the increase in light chains 1s and 2s followed symmetrical time courses such that the ratio between the alkali and the DTNB light chains remained constant. (Therefore, myosin molecules

containing less than 4 light chains do not exist at any time during the transformation). They have suggested that the stoichiometric control exists at the level of transcription.

In terms of heavy chain changes, Sreter et al., (1974), noted a marked difference in the subperiodicity and staining pattern of the light meromyosin paracrystals. In subsequent work, these authors (Sreter et al., 1975) found that the concentration of N-methylhistidine (an amino acid found in the heavy chains of fast fibres) decreased with stimulation suggesting a replacement of the heavy chains of fast muscle by heavy chains normally associated with slow muscle.

Many of these studies have confirmed that there is a gradual transformation of fibre type throughout the course of stimulation rather than replacement of one fibre type by another. Eisenberg and Salmons (1981) using fibre-specific myosins demonstrated that fibres stimulated for intermediate periods of time contained a mixture of slow and fast myosin light heavy chains. Fibres which had been completely transformed through electrical stimulation were shown to return to their original histochemical characteristics upon removal fo the stimulation (Eisenberg and Salmons, 1981). Lastly, Helig and Pette (1983) did in vitro translation of mRNA from stimulated muscle and revealed the co-existence of mRNA coding for fast type and mRNA coding for slow type muscle.

*excluded due to copyright restrictions

Figure A.3: Time course of electrophoretically determined specific peptides of SR from fast-twitch rabbit muscle during chronic nerve stimulation. Values are expressed as ratios between stimulated and contralateral muscles. (Wiehrer and Pette, 1983).

Table A.2: Relative time course of stimulation-induced changes in animal skeletal muscle.

PARAMETER	TIME	REFERENCE
Parvalbumin ⇩	6 days	9
SR: ⇩ Ca^{2+} ATPase activity	2 days	13
⇩[115 kdal] protein	3 days	13
SDS gel pattern	3 days	16
	2 days	19
⇧Capillarization	4 days	38,40
Enzymes and isozymes	4-14 days	1,13
⇧ Z-disc thickness	2 weeks	43
	1.5-2.5 weeks	44
⇧time to peak tension	up to 14 days	13
and half relaxation time		
Tropomyosin	3 weeks	31
Myosin ATPase:		
Light chain	4 weeks	45
Heavy chain	60-90 days	45

Note. For numerical references see end of Chaper 1.

amount of time as the 24 hrs/day protocol.

d) Time Course of Stimulation-Induced Changes

An orderly sequence of change has been documemted in the various structural and functional domains. Figure A.3 illustrates the time course of some SR peptides in response to chronic stimulation as summarized by Wiehrer and Pette (1983). Table A.2 is a relative time course for stimulation induced changes in various metabolic, functional and contractile changes. Positions on the continuum are relative as the various studies of the time course of adaptations used protocols which differed in the daily dose of stimulation. For example, Pette et al., (1976) in a chronic stimulation study, used both continuous and intermittent protocols. They observed the same ultimate changes, but the intermittent protocol (8 hrs/day) required 3 times the amount of time as the 24 hrs·day^{-1} protocol.

e) Nature of the Stimulus

Various protocols for the application of electrical stimulation allow one to differentiate between the pattern of stimulation and total impulse activity as the underlying cause of fibre transformations.

Initially it was proposed that the fibre composition was dictated by a chemical message from the motor nerve. However, the fact that cross re-innervation and electrical stimulation generate similar results argued for a role of the pattern of nervous activity. Salmons and Sreter (1976) found that the

effects of the two protocols were mediated in the same way and ruled out a chemotrophic factor. Salmons and Vrbova (1969) and Brown et al., (1976) applied low frequency and high frequency stimulation protocols and observed that a stimulus pattern equivalent to the post stimulation fibre type was required to institute a transformation. For example, Brown et al., (1976) used continuous low frequency stimulation and intermittent high frequency stimulation and observed changes in metabolic and ultrastructural profiles only with the former protocol. Likewise, Pette et al., (1973) observed comparable fibre transformations at a particular stimulation frequency, whether the muscle was stimulated continuously or intermittently. Thus, these researchers argued that the key factor is the pattern of stimulation. However, these authors did not equate the total amount of contractile activity when setting up the different scenarios. Many other investigatiors observed equivalent changes in contraction time and fatiguability using a variety of frequency patterns, provided the total amount of contractile activity was equalized (Hudlicka and Tyler, 1980; Sreter et al., 1982; Hudlicka et al., 1982b). In addition, Jolesz et al., (1981) and Sreter et al., (1982) observed that inactivity through either cordotomy or chronic immobilization lead to a slow to fast fibre type conversion. Salmons et al.,(1982) further confirmed the role of total activity when they observed that a fast type muscle could be transformed to a slow type muscle through

intermittent stimulation at a stimulation pattern physiologically closer to a fast muscle than a slow mluscle. The fact that total neural impulse and therefore contractile activity plays a dominant role in the transformation process, sets the stage for the use of exercise in an attempt to achieve conversion of fibre types thought what may be considered a more physiological stimulus.

Helig et al., (1984) observed that chronic stimulation increased the total RNA content of rabbit tibialis anterior to the level of soleus muscle within 8 days. These authors suggested that the transformation process involves independent changes in both transcription and translation. Transcriptional control was evident form the detection of mRNA and its translation products in vivo. Differences between in vivo and <u>in vitro</u> translations were recorded and it was therefore concluded that translational control must also be in effect.

High intensity training programs and skeletal muscle fibre composition in animals

The fact that total neural impulse and therefore total contractile activity are central to fibre type transformation after electrical stimulation, suggests that such interconversion of muscle fibre types may occur in response to an exercise stimulus.

Green et al., (1983) observed changes in the enzyme profile of skeletal muscle which were quantitatively comparable to those reported after electrical stimulation. This particular study employed a high intensity long-term endurance running protocol. They observed increases in the activity of aerobic enzymes and decreases in the enzymes of glycolysis and glycogenolysis (although in the case of Phosphofructokinase and Glycogen Phosphorylase the change was limited to the type IIA fibres). Both Baldwin et al., (1977) and Terjung (1976) highlighted the role of recruitment pattern in these experiments which does not appear to be a factor in studies involving electrical stimulation. Terjung (1976) pointed out that IIB fibres are called upon at greater treadmill inclines irrespective of the pace of running. The animals were observed to increase their speed of contraction only moderately over a wide range of running speeds. Thus, adaptation to a particular intensity was suggested to account for the transient response of Hexokinase (Baldwin et al.,

1977).

Changes in skeletal muscle SR structure and function in response to intense exercise have included decreased parvalbumin concentration and changes in the peptide pattern of some SR membrane proteins. Green et al., (1984) observed differences between the deep (predominantly IIA fibres (Baldwin et al., 1972)) and superficial (predominantly IIB fibres) portions of the vastus lateralis, presumably due to the recruitment pattern described above. A decrease in the ratio of the 115 kdal / 30 kdal proteins, a phenomenon reported to occur after electrical stimulation was manifested as a decrease in the concentration of the 115 kdal Ca^{2+} ATPase protein and an increase in the concentration of the 30 kdal protein. Belcastro and Wenger (1982) observed an increase in total calcium uptake from training in contrast to the results observed in studies employing electrical stimulation. This study however, involved steady state training rather than a protocol attempting to induce fibre type interconversion. Bonner et al., (1976) also observed an increase in calcium sequestration by the SR in response to training. They accounted for this as an adaptive response to exercise, such that the SR would take over the role of $[Ca^{2+}]$ regulation from the mitochondria (in slow muscle) inorder to facilitate ATP production, a proceedure inhibited by high concentrations of calcium within the mitochondria. Therefore, the results of the Belcastro and Wenger (1982) study must be interpreted with

the protocol in mind.

Green et al., (1984) did observe changes in the contractile machinery. In the deep vastus lateralis (IIA fibres) only, significant increases were observed in the electrophoretic concentrations of 1s and 2s light chains with corresponding increases in 1f and 3f chains. Like the studies employing electrical stimulation the ratio of alkali to DTNB light chains remained stable (Green et al., 1984).

APPENDIX B

LABORATORY PROCEDURES

APPENDIX B-1

PROTEIN DETERMINATION FOR TOTAL MUSCLE PROTEIN

(Lowry et al., 1951; as modified by Campbell and Sargeant, 1967)

Reagents and Chemicals

1. 0.5% Cupric Sulfate ($CuSO_4 \cdot 5H_2O$)
2. 1.0% Sodium Potassium Tartrate ($NaKC_4H_4O_6$)
3. 2.0% Sodium Carbonate (Na_2CO_3), pH to 12.5 with 10 N NaOH at room temperature
4. Lowry C solution: 61% $NaCo_3$
   35.7% deionized H_2O
   1.3% $CuSo_4 \cdot 5H_2O$
   1.3% $NaKC_4H_4O_6$
5. Folin Reagent: 1 to 1 (v/v) with deionized water
6. 0.3 N NaOH
7. Suspension medium (see S.R. Isolation)

Standard Curve (Protein stock solution: 5 $mg \cdot ml^{-1}$ of bovine serum albumin in deionized water)

stock (ml)	suspension medium (ml)	conc1 ($mg \cdot ml^{-1}$)	conc 2 ($ug \cdot ml^{-1}$)
0.00	0.50	0.00	0.00
0.05	0.45	0.50	3.00
0.10	0.40	1.00	6.00
0.20	0.30	2.00	12.00
0.30	0.20	3.00	19.00
0.40	0.10	4.00	25.00
0.50	0.00	5.00	30.00

Procedure

1. Solubilizing Protein

a) Take 0.1 ml of homogenate or standard solution.

b) Add 0.2 ml of 0.3 N KOH.

c) Incubate in water bath at 37° for 30 minutes.

2. Reaction Mixture

a) Take 0.1 ml (twice) of solubilized protein from above.

b) Add 5.0 ml of freshly prepared Lowry C solution to duplicate tubes.

c) Add 0.3 ml of folin reagent to each tube while vortexing. Insure equal mixing for all tubes.

d) Allow reaction mixture to stand for at least 45 minutes.

3. Spectrophotometric Analysis

a) Using the pye Unicam PU 8800 UV/VIS spectrophotometer at a wavelength of 750 nm, record the optical density. Use the protein blank from the standard curve as the reference and vortex each standard and sample tube before its analysis.

APPENDIX B - 2

Protein Determination for Membrane Systems

(Lowry et al., 1951)

Reagents and Chemicals

1. Stock Solution A1: 2% Sodium Carbonate (Na_2CO_3)
   0.1 N Sodium Hydroxide (NaOH)
2. Stock Solution A2: 10% Sodium Dodecyl Sulfate (SDS)
   (prepared fresh daily)
3. Stock Solution B1: 1% Cupric Sulfate ($CuSo_4 \cdot 5H_2O$)
4. Stock Solution B2: 2% Sodium Potassium Tartrate
   ($NaKC_4H_4O_6$)
5. Lowry C solution: 50.0 ml stock solution A1
   5.0 ml stock solution A2
   0.5 ml stock solution B1
   0.5 ml stock solution B2
6. Folin Reagent: 1 to 1 (v/v) with deionized water

Standard Curve (Protein stock solution: 1 $mg \cdot ml^{-1}$ of bovine serum albumin in deionized water)

stock (ml)	H_2O (ml)	conc 1 ($mg \cdot ml^{-1}$)	conc 2 ($ug \cdot ml^{-1}$)
0.000	0.500	0.000	0.000
0.025	0.475	0.050	0.010
0.050	0.450	0.100	0.020
0.100	0.400	0.200	0.040
0.150	0.350	0.300	0.060
0.200	0.300	0.400	0.080
0.250	0.250	0.500	0.100

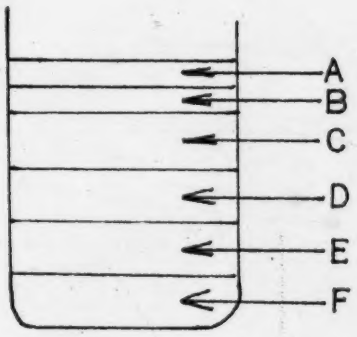
Proceedure

1. Transfer 2 x 0.100 ml aliquots of each tube (at concentration 1) to duplicate tubes containing 0.4 ml H_2O, resulting in the concentration listed as "2."

2. Add 3 ml of Lowry solution C to each duplicate tube. Allow the tubes to sit for 10 minutes or more.

3. Add 0.3 ml of the diluted Folin Reagent to each tube, while vortexing. Allow the tubes to sit for 30 minutes or more.

4. Using the Pye Unicam PU 8800 UV/VIS spectrophotometer, record the optical density at a wavelength of 750 nm. Use the protein blank from the standard curve as the reference.



APPENDIX B-3

Isolation of SR by Density Gradient Centrifugation

(Saito et al., 1984)

Reagents and Chemicals

1. Homogenization Buffer A: 300 mM Sucrose
   20 mM Imidazole HCl
   pH = 7.4 0.5 mM EDTA
   0.5 mM DTT

2. Homogenization Buffer B: 300 mM Sucrose
   20 mM Imidazole HCl
   pH = 7.4 0.5 mM Mg ATP
   0.5 mM DTT

3. Suspension Buffer A: 300 mM Sucrose
   5.0 mM Imidazole HCl
   pH = 7.4 0.5 mM DTT

Proceedure

1. Grind whole muscle tissue to a powder, under liquid N_2.
2. Homogenize the ground tissue to 5 volulmes of homogenization buffer A. Using the Brinkmann polytron, set at 4.5, homogenize twice for 15 seconds each.
3. Using the JA-20 Beckman rotor, centrifuge for 10 minutes at 8,000 rpm.
4. Decant and retain supernatant (S#1), through 4 layers of

cheesecloth.

5. Resuspend pellet (P#1) in 5 volumes of homogenization buffer B.

6. At a polytron setting of 4.5, homogenize the resuspended pellet twice, for 15 seconds each.

7. Using a JA-20 Beckman rotor, centrifuge for 10 minutes at 8,000 rpm.

8. Decant and retain the supernatant (S#2), through 4 layers of cheesecloth.

9. Using a Beckman Ti-60 rotor, cnetrifuge for 45 minutes (42 + 3) at 40,000 rpm.

10. Vacuum aspirate and discard the supernatant.

11. Resuspend the pellet in 0.75 ml of suspension buffer A and quick freeze in liquid N_2.

12. Thaw the frozen resuspended pellet and layer on the sucrose gradient as shown.

where A: 0.75 ml of 5 mM Imidazole HCl

B: pellet, thawed and resuspended

C: 7 ml of (1.76 M sucrose + 0.5 mM DTT)

D: 8 ml of (1.16 M sucrose + 0.5 mM DTT)

E: 7 ml of (1.40 M sucrose + 0.5 mM DTT)

F: 6 ml of (1.72 M sucrose + 0.5 mM DTT)

13. Using Beckman rotor SW-28, centrifuge for 16 hours at 20,000 rpm.

14. Resuspend fractions D and E in 5 mM Imidazole HCl to 25 ml total (8 tubes).

15. Using Beckman rotor Ti-60, centrifuge for 25 minutews at 40,000 rpm.

16. Resuspend the pelets in 0.5 ml of suspension buffer A.

17. Divide in 50 ul aliquots and quick freeze in liquid N_2. Store at -70·C until future use.

APPENDIX B-4

ATPase Enzyme Activity

(Parkhouse and Belcastro, 1987)

Reagents and Chemicals

1. Standard Reaction Media: 300 mM Sucrose
   100 mM KCl
   10 mM PIPES
   7.5 mM $MgCl_2$

Using the Standard Reaction Media, make up the following solutions, such that the listed concentration represents that within the spectrophotometric cuvette.

a) 1 mM EGTA solutions

b) 20 mM Ca^{2+} solution

c) 1 mM EGTA
   10 uM NaN_3 in solution

d) 1 mM EGTA
   5 uM Ruthenium Red (RR) in solution

2. Using deionized water as the solvent, make up the following solutions, such that the listed concentration represents that within the spectrophotometric cuvette.

a) 2 mM PEP

b) 0.2 mM NADH

c) 10 U LDH

d) 10 U PK

e) 5 mM Mg ATP

Procedure

1. Dilute the thawed SR protein to 1 $mg \cdot ml^{-1}$.

2. In a 1 ml cuvette incubate the following in a 37 · C water bath for 10 minutes.

.10 ml PEP

.04 ml NADH

.01 ml LDH

.01 ml PK

.50 ml Standard Reaction Media

.14 ml SR Suspension Buffer A

.05 ml protein

.05 ml variable standard solutions, (ie Ca^{2+} solution or EGTA·RR solution)

3. Using the Pye Unicam PU 8800 UV/VIS spectrophotometer at a wavelength of 340 nm, establish and record a baseline optical density. Use a chart speed of 10 $s \cdot cm^{-1}$.

4. Begin a timed recording of the change in optical density with time upon addition of 0.10 ml Mg ATP. Terminate with perchloric acid.

5. Use the following equation to calculate enzyme activity in units of umol / mg protein / minute.

$$\frac{OD}{SEC} \times \frac{\text{CUVETTE VOLUME}}{\text{SAMPLE (PROTEIN) VOLUME}} \times \frac{60\ S}{6.22}$$

APPENDIX B-5

Chemicals and their Source

Cupric Sulfate	Fisher
Sodium Potassium Tartrate	Fisher
Sodium Carbonate	BDH
NaOH	BDH
Folins Reagent	Sigma
BSA	Sigma
SDS	BDH
Sucrose	BDH
Imidazole HCl	Fisher
EDTA	Fisher
EGTA	Sigma
DTT	Sigma
KCl	Fisher
PIPES	Sigma
$MgCl_2$	Fisher
Mg-ATP	Sigma
NaN_3	Fisher
RR	Fluka
PEP	Boehringer Mannheim
NADH	Boehringer Mannheim
LDH	Boehringer Mannheim
PK	Boehringer Mannheim
KOH	Chemonics Scientific
CaCl2	Fisher

APPENDIX C

Statistics Summary

SR YIELD:

	SS	DF	MS	F	P
Training effect (T)	7225.00	1	7225.00	9.95	<.01
Vesicle Type (VT)	7832.25	1	7832.25	10.79	<.01
T X VT	2500.00	1	2500.00	3.44	ns
Within	8711.20	12	725.93		

---

ns = not significant

TOTAL ($Mg2^{+}$, Ca^{2+} ATPase) ACTIVITY:

	SS	DF	MS	F	P
Training Effect (T)	1.85	1	1.85	48.94	<.01
Vesicle Type (VT)	3.92	1	3.92	124.44	<.01
T X VT	1.12	1	1.12	35.55	<.01
Within	0.378	12	0.0315		

Post-hoc T X VT t-test yielded a 't' of 22.92, $p<.01$

Ca^{2+} ATPase ACTIVITY:

	SS	DF	MS	F	P
Training Effect (T)	1.96	1	1.96	5.63	<.05
Vesicle Type (VT)	3.86	1	3.86	11.09	<.01
T X VT	1.03	12	0.086	0.247	ns
Within	0.348				

Post-hoc T X VT t-test yielded a t of 12.024, $p<.01$

TOTAL MUSCLE PROTEIN:

	SS	DF	MS	F	P
Training Effect (T)	9.44	1	9.44	0.076	ns
Muscle Type (MT)	777.74	1	777.74	6.2648	<.05
T X MT	124.85	1	124.85	1.006	ns
Within	3103.62	25	124.14		

Summary of t-tests to invesitgate the effect of NaN_3 and RR

VESICLE TYPE	t	p
1. NaN_3		
Control-light	.043	ns
control-heavy	.018	ns
Exercise-light	.023	ns
Exercise-heavy	.037	ns
2. RR		
Control-light	.096	ns
Control-heavy	.026	ns
Exercise-light	.041	ns
Exercise-heavy	.141	ns

(critical values of p: 3.18 for alpha of .05
5.84 for alpha of .01)

ns = not significant

Summary of t-test for select anthropometric data:

	t	p
Terminal Body Weight*	.624	ns
Heart Weight	.421	ns
HW:BW ratio	.476	ns
Muscle Mass		
Epitroclearis	.109	ns
Tibialis Anterior	.124	ns
Normalized Muscle Mass		
Epitroclearis	.169	ns
Tibialis Anterior	.151	ns

---

ns = not significant

* value for terminal body weight is an F ratio for the multivariate ANOVA shown on the following page.

Summary of post-hoc t-tests:

	t	significance
Ca^{2+} Mg^{2+} ATPase activity:		
Control light vs heavy	4.88	p<.01
Trained light vs heavy	16.32	p<.01
Heavy trained vs control	11.92	p<.01
Light trained vs control	2.06	ns
Ca^{2+} ATPase activity:		
Control light vs heavy	4.96	p<.01
Trained light vs heavy	16.15	p<.01
Heavy trained vs control	11.23	p<.01
Light trained vs control	2.27	ns
SR Yield:		
Control light vs heavy	4.28	p<.01
Trained light vs heavy	0.90	ns
Heavy control vs trained	3.32	ns
Light control vs trained	1.01	ns

Mean body weight (g) for control and training groups at the end of each training week. Mean food consumed (mg·g body weight $^{-1}$) for each week.

	MEAN BODY WEIGHT			MEAN FOOD CONSUMED		
WEEK	CONTROL	TRAINING	SIG.	CONTROL	TRAINING	SIG.
1	174.885	176.429	ns	72.762	72.100	ns
2	182.250	174.429	ns	81.450	80.371	ns
3	188.750	186.286	ns	81.575	86.629	ns
4	193.625	193.286	ns	78.500	88.757	p<.001
5	200.750	202.857	ns	77.287	92.700	p<.005
6	208.625	207.857	ns	81.750	97.986	p<.001
7	210.125	211.714	ns	81.300	92.614	p<.001
8	212.500	214.143	ns	75.725	87.643	p<.001
9	216.750	217.429	ns	78.800	93.243	p<.001
10	224.375	221.571	ns	77.550	86.300	p<.005
11	225.375	220.857	ns	76.537	87.086	p<.005
12	230.250	227.714	ns	72.175	87.071	p<.001
13	231.250	223.143	ns	70.762	82.329	p<.005
14	236.500	226.571	ns	67.000	84.786	p<.001
15	237.625	228.857	ns	66.925	90.429	p<.001
16	244.875	237.857	ns	66.050	80.071	p<.001

HIERARCHICAL SUMMARY TABLE OF F-RATIOS FOR: WT1

TYPE	PART OF MODEL		SSH	SSE	MSH	MSE	F-RATIO	DFH	DFE	PROB
UNIV	GRAND MEAN		.10814108	49323.50	10814108	3794.12	2850.24	1.0	13.0	0.0
UNIV	GROUP —		526.46	49323.50	526.46	3794.12	0.14	1.0	13.0	0.7155
UNIV	CASES(GROUP)	*	49323.50	6763.79	3794.12	34.69	109.38	13.0	195.0	0.0
ERROR TERM: CASES(GROUP)										
UNIV	WEEK —		90607.80	6763.79	6040.52	34.69	174.15	15.0	195.0	0.0
GREENHOUSE-GEISER ADJ:			EPSILON:	0.25	6040.52	34.69	174.15	3.8	49.0	0.0
UNIV	GROUP*WEEK —		987.86	6763.79	65.86	34.69	1.90	15.0	195.0	0.0253
GREENHOUSE-GEISER ADJ:			EPSILON:	0.25	65.86	34.69	1.90	3.8	49.0	0.1292
UNIV	CASES(GROUP)	*	49323.50	6763.79	3794.12	34.69	109.38	13.0	195.0	0.0
UNIV	WEEK*CASES(GROUP)	*	6763.79	****	34.69	****	****	195.0	****	****
ERROR TERM: WEEK*CASES(GROUP)										

HIERARCHICAL SUMMARY TABLE OF F-RATIOS FOR: FC1

TYPE	PART OF MODEL		SSH	SSE	MSH	MSE	F-RATIO	DFH	DFE	PROB
UNIV	GRAND MEAN		.15647007	2146.84	.15647007	165.14	9474.96	1.0	13.0	0.0
UNIV	GROUP		7879.51	2146.84	7879.50	165.14	47.71	1.0	13.0	0.0
UNIV	CASES(GROUP)	*	2146.84	5109.56	165.14	26.20	6.30	13.0	195.0	0.0
ERROR TERM: CASES(GROUP)										
UNIV	WEEK		5368.86	5109.56	357.92	26.20	13.66	15.0	195.0	0.0
GREENHOUSE-GEISER ADJ:			EPSILON:	0.35	357.92	26.20	13.66	5.3	68.5	0.0
UNIV	GROUP*WEEK		2254.77	5109.56	150.32	26.20	5.74	15.0	195.0	0.0
GREENHOUSE-GEISER ADJ:			EPSILON:	0.35	150.32	26.20	5.74	5.3	68.5	0.0001
UNIV	CASES(GROUP)	*	2146.84	5109.56	165.14	26.20	6.30	13.0	195.0	0.0
UNIV	WEEK*CASES(GROUP)	*	5109.56	****	26.20	****	****	195.0	****	****
ERROR TERM: WEEK*CASES(GROUP)										

EFFECT .. CONSTANT

Univariate F-tests with (1,13) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
WT1	460746.34405	2060.58929	460746.34405	158.50687	2906.78104	.000
WT2	474853.18571	1718.21428	474853.18571	132.24726	3591.40304	.000
WT3	525100.00476	2626.82857	525100.00476	202.07143	2598.88810	.000
WT4	558878.62976	3201.30357	558878.62976	246.25412	2268.52387	.000
WT5	608186.24286	3642.35714	608186.24286	280.18132	2170.57742	.000
WT6	647674.20119	3486.73214	647674.20119	268.67170	2428.35738	.000
WT7	664340.62976	3736.30357	664340.62976	287.40787	2311.48898	.000
WT8	678858.74286	3736.65714	678858.74286	287.48055	2364.08228	.000
WT9	703774.51905	3781.21428	703774.51905	291.63187	2413.22813	.000
WT10	742441.34405	4078.58929	742441.34405	313.81456	2385.88009	.000
WT11	743383.00119	3542.73214	743383.00119	272.51786	2727.88895	.000
WT12	762886.80476	4216.92857	762886.80476	324.37912	2413.83232	.000
WT13	770632.04286	4366.35714	770632.04286	337.41208	2284.54187	.000
WT14	800557.88571	3829.71428	800557.88571	294.58341	2717.50103	.000
WT15	812394.20119	4274.73214	812394.20119	328.13324	2488.28288	.000
WT16	889878.88786	3771.73214	889878.88786	290.13324	2986.65288	.000
FC1	78344.53083	765.18882	78344.53083	58.86145	1330.99804	.000
FC2	97761.70380	668.27447	97761.70380	51.48265	1898.92520	.000
FC3	105625.10431	506.64917	105625.10431	38.12886	2698.85488	.000
FC4	104438.80270	115.01728	104438.80270	8.84748	11804.48418	.000
FC5	107877.45383	914.80681	107877.45383	70.36451	1533.34103	.000
FC6	120336.78126	386.68826	120336.78126	29.78064	4043.48843	.000
FC7	112818.05381	361.84852	112818.05381	27.83450	4056.80178	.000
FC8	98639.12837	377.01212	98639.12837	29.00093	3435.72157	.000
FC9	110501.88255	223.65724	110501.88255	17.19871	6425.78143	.000
FC10	100226.12480	322.45995	100226.12480	24.80461	4040.70529	.000
FC11	98850.88057	482.82731	98850.88057	37.14825	2660.59371	.000
FC12	94675.17123	532.32827	94675.17123	40.94441	2312.06002	.000
FC13	87487.65386	440.57312	87487.65386	33.88024	2581.78504	.000
FC14	86011.88870	355.38865	86011.88870	27.33605	3148.48368	.000
FC15	82437.88698	356.94908	82437.88698	27.45762	3388.58483	.000
FC16	78712.14760	443.73448	78712.14760	34.13342	2335.31077	.000

EFFECT .. GROUP

Univariate F-tests with (1,13) D. F.

Variable	Hypoth. SS	Error SS	Hypoth. MS	Error MS	F	Sig. of F
WT1	9.01071	2060.58929	9.01071	158.50687	.05685	.816
WT2	228.38571	1718.21429	228.38571	132.24726	1.72696	.212
WT3	22.67143	2626.92857	22.67143	202.07143	.11220	.743
WT4	.42976	3201.30357	.42976	246.25412	.00175	.967
WT5	16.57619	3642.25714	16.57619	280.18132	.05916	.812
WT6	2.20119	3486.73214	2.20119	268.67170	.00820	.929
WT7	9.42976	3736.30357	9.42976	287.40797	.03281	.858
WT8	10.07619	3736.85714	10.07619	287.45055	.03505	.854
WT9	1.71905	3781.21429	1.71905	291.63187	.00589	.940
WT10	28.34405	4079.58929	28.34405	313.81456	.09351	.766
WT11	76.20119	3542.73214	76.20119	272.51786	.27962	.606
WT12	24.00476	4216.82857	24.00476	324.37912	.07400	.790
WT13	245.37619	4336.38714	245.37619	337.41208	.72723	.408
WT14	388.01905	3828.71429	388.01905	294.68341	1.24824	.284
WT15	287.00119	4276.73214	287.00119	328.13324	.87188	.367
WT16	183.86786	3771.73214	183.86786	290.13324	.63374	.440
FC1	1.63860	765.19882	1.63860	58.86145	.02784	.870
FC2	4.34304	889.27447	4.34304	51.48285	.08436	.778
FC3	95.34387	508.64817	95.34387	39.12586	2.43678	.143
FC4	392.77989	115.01728	392.77989	8.84748	44.39454	.000
FC5	885.83526	914.60861	885.83526	70.35451	12.60524	.004
FC6	880.00483	385.88828	880.00483	29.78064	32.25754	.000
FC7	477.81564	361.84852	477.81564	27.83450	17.16990	.001
FC8	530.28525	377.01212	530.28525	29.00093	18.28442	.001
FC9	778.75836	223.55724	778.75836	17.19671	45.28531	.000
FC10	285.83326	322.45995	285.83326	24.80461	11.52339	.005
FC11	415.38848	482.92731	415.38848	37.14825	11.18181	.005
FC12	828.44004	532.32927	828.44004	40.94841	20.23131	.001
FC13	489.42319	440.57312	489.42319	33.89024	14.73648	.002
FC14	1180.97157	355.38865	1180.97157	27.33805	43.20198	.000
FC15	2062.35870	356.84809	2062.35870	27.45762	75.11085	.000
FC16	733.87604	443.73448	733.87604	34.13342	21.50312	.000

APPENDIX D

RAW DATA

APPENDIX D-1

Raw Data: Body Mass (grams)

	CONTROLS								RUNNERS							
DATE	04	08	12	13	18	21	35	38	10	17	19	23	26	36	39	
31.7	188	177	185	188	137	164	168	190	171	163	182	175	201	183	184	
06.8	186	168	183	190	145	165	169	185	169	160	178	164	185	177	175	
08.8	183	177	181	193	144	166	168	187	171	166	182	171	190	180	175	
11.8	183	186	190	193	150	174	172	188	174	169	175	174	190	181	174	
13.8	187	180	182	196	149	173	168	191	157	170	181	173	180	171	181	
15.8	190	185	191	201	153	175	172	191	173	167	179	176	170	180	176	
18.8	193	191	194	205	153	181	166	194	169	173	187	179	194	182	188	
20.8	194	190	195	201	151	178	176	201	175	173	187	176	203	189	184	
22.8	196	197	199	205	154	183	177	199	172	178	194	181	203	190	186	
25.8	202	198	201	210	156	186	183	207	180	178	198	188	208	192	185	
27.8	200	202	205	211	158	185	187	206	186	189	170	188	208	199	183	
29.8	200	199	203	211	154	187	184	211	189	198	181	193	215	192	185	
01.9	206	202	211	213	157	194	192	216	194	198	190	195	217	199	192	
03.9	207	207	211	218	160	195	197	214	200	203	190	200	228	205	196	
05.9	208	209	215	215	160	188	196	215	200	206	187	193	226	213	195	
08.9	208	214	217	228	163	200	202	221	199	202	188	201	226	211	195	
10.9	214	209	216	218	164	196	200	221	203	202	192	201	208	210	203	
12.9	210	215	219	226	168	202	204	225	212	199	190	203	226	223	202	
15.9	220	215	215	224	162	196	202	223	216	208	193	200	224	219	209	
17.9	217	214	221	221	167	201	205	226	220	206	190	207	230	214	211	
19.9	220	220	221	227	165	202	201	225	223	216	194	207	226	211	205	
22.9	223	229	221	229	170	204	209	228	214	219	194	210	231	213	208	
24.9	220	223	221	226	170	205	205	227	221	219	192	214	236	220	207	
26.9	221	221	220	228	170	201	208	231	217	214	193	214	234	222	205	
29.9	223	222	225	235	171	205	212	232	229	221	198	211	241	229	212	
01.10	225	223	224	235	173	204	214	235	223	227	192	214	239	231	209	
03.10	227	228	224	232	173	205	211	234	213	237	200	214	228	220	210	
06.10	230	226	229	240	175	211	218	234	219	241	203	224	236	228	217	
08.10	231	226	230	240	175	213	214	239	215	227	201	228	224	220	213	
10.10	236	230	236	245	177	214	219	238	222	233	199	226	231	224	216	
13.10	237	233	227	242	177	217	222	240	223	224	196	229	237	227	217	
15.10	241	233	234	247	177	213	222	244	219	228	202	222	239	228	219	
17.10	234	234	231	245	181	218	218	242	218	227	202	225	235	220	219	
20.10	237	239	233	251	179	219	226	246	224	243	212	230	228	231	236	
22.10	237	239	232	251	187	224	219	242	234	241	211	232	228	232	233	
24.10	241	238	237	253	181	224	220	248	227	235	207	235	234	231	225	

27.10	244	244	232	252	183	226	218	250	235	237	207	228	236	233	228
29.10	243	239	238	256	185	227	225	247	232	235	205	218	243	226	224
31.10	245	238	237	252	183	225	217	253	223	222	204	218	234	234	227
03.11	245	237	245	259	187	226	218	251	227	234	209	228	245	227	232
05.11	246	239	241	253	184	226	217	252	226	227	196	219	241	229	221
07.11	247	241	247	261	192	231	222	251	232	228	207	231	237	228	223
10.11	252	241	241	254	190	230	222	253	238	240	204	231	240	222	222
12.11	249	240	245	259	187	228	224	257	244	239	206	233	234	221	226
14.11	256	238	239	261	191	234	227	255	241	233	207	225	238	224	234
17.11	259	248	248	269	189	229	227	258	234	235	212	220	237	234	233

APPENDIX D-2
Raw Data: Food Consumption (grams)

	CONTROLS								// RUNNERS						
DATE	04	08	12	13	18	21	35	38	//10	17	19	23	26	36	39
08.08	24	29	23	27	23	26	07	26	29	26	27	24	24	27	20
11.08	43	46	51	46	42	44	38	44	42	43	37	46	46	40	39
13.08	31	24	27	31	23	27	24	30	13	27	32	26	20	20	29
15.08	31	31	37	35	28	32	27	31	38	30	28	34	10	29	28
18.08	51	57	52	48	41	43	32	47	41	43	47	43	55	50	49
20.08	29	30	35	31	26	28	27	32	31	36	37	31	42	32	26
22.08	31	36	35	33	29	28	23	29	28	33	36	33	31	33	32
25.08	49	48	53	52	41	45	44	51	sp	52	50	55	65	53	47
27.08	30	35	34	35	27	28	30	31	47	32	16	32	33	32	36
29.08	29	27	33	30	22	29	26	32	39	33	34	31	41	35	23
01.09	49	56	53	48	41	43	43	52	60	57	72	62	63	64	57
03.09	33	32	32	37	25	28	26	30	39	33	33	36	45	37	33
05.09	30	37	36	34	27	23	28	30	41	46	sp	32	37	48	36
08.09	54	58	62	59	48	48	51	55	59	57	55	55	66	62	53
10.09	35	33	36	29	22	27	26	30	44	35	36	46	21	44	35
12.09	34	38	36	39	30	34	34	38	48	35	35	42	47	50	37
15.09	58	57	49	51	47	41	46	49	73	57	56	51	65	65	65
17.09	34	35	39	40	27	35	30	30	49	36	33	44	38	36	41
19.09	37	46	34	33	25	33	37	34	46	42	37	39	39	37	36
22.09	50	62	55	51	45	46	47	sp	55	63	53	60	63	58	54
24.09	32	35	34	34	27	31	28	30	39	38	37	39	36	45	35
26.09	31	30	35	35	29	27	30	33	35	34	33	41	44	42	29
29.09	55	56	62	57	44	47	52	51	73	59	59	61	67	71	63
01.10	32	31	31	38	37	27	29	41	30	38	32	40	44	47	35
03.10	39	40	40	37	23	32	32	32	39	48	44	43	34	40	42
06.10	56	49	49	56	45	50	50	43	58	72	56	69	63	59	65
08.10	36	35	35	36	31	34	30	39	35	29	33	39	30	34	38
10.10	37	38	38	38	29	33	sp	32	40	37	35	39	36	40	38
13.10	59	56	56	58	49	52	52	65	59	49	53	69	73	65	61
15.10	37	40	34	38	30	33	29	36	30	36	39	43	47	41	39
17.10	30	33	31	33	28	28	29	31	36	33	37	39	34	32	39
20.10	53	55	53	61	46	50	48	54	66	71	75	73	66	63	70
22.10	31	33	35	34	35	32	26	29	42	39	sp	43	28	40	39
24.10	37	36	33	35	27	30	25	33	35	32	30	40	35	37	34
27.10	54	53	50	52	44	47	42	52	66	57	55	56	58	73	60
29.10	36	33	36	36	31	33	29	34	39	33	32	30	42	36	37
31.10	36	30	38	33	28	29	26	34	31	28	37	36	39	41	38
03.11	49	48	55	52	45	47	39	48	57	60	56	67	70	58	60
05.11	32	34	34	30	26	27	27	31	34	35	26	29	41	45	32
07.11	33	32	33	35	30	29	31	31	44	29	40	53	41	37	36
10.11	54	50	51	50	45	48	43	48	64	68	56	72	63	64	58
12.11	34	32	31	33	27	28	29	34	45	39	39	46	36	41	41
14.11	sp	30	35	32	28	32	26	33	39	32	40	41	42	45	42
17.11	53	52	52	52	41	46	39	46	48	53	55	56	60	57	56

Note. These are 2-day and 3-day consumptions. sp= spilled food

APPENDIX D-3
Raw Data: Work load and Power Output

WEEK	DATE	WORK (kg m)	POWER (watts)
1	6.8.86	8.040	.0730
1	8.8	10.043	.0746
2	11.8	12.966	.0815
2	13.8	15.891	.0866
2	15.8	17.058	.0871
3	18.8	22.719	.0977
3	20.8	27.220	.1059
3	22.8	30.202	.1073
4	25.8	35.679	.1166
4	27.8	40.791	.1232
4	29.8	44.743	.1261
5	1.9	51.824	.1366
5	3.9	59.735	.1479
5	5.9	63.293	.1477
6	8.9	70.465	.1556
6	10.9	77.793	.1630
6	12.9	83.881	.1667
7	15.9	103.278	.1688
7	17.9	112.178	.1697
7	19.9	120.830	.1702
8	22.9	131.496	.1791
8	24.9	143.910	.1896
8	26.9	147.518	.1883
9	29.9	163.094	.2019
9	1.10	174.292	.2094
9	3.10	DID NOT RUN - EQUIPMENT DYSFUNCTION SCHEDULE SET BACK THREE DAYS	
10	6.10	180.646	.2139
10	8.10	188.276	.2166
10	10.10	196.505	.2199
11	13.10	209.796	.2285
11	15.10	223.715	.2374
11	17.10	232.622	.2406
12	20.10	250.901	.2531
12	22.10	266.920	.2627
12	24.10	270.503	.2600
13	27.10	287.630	.2701
13	29.10	299.520	.2749
13	31.10	302.180	.2713
14	3.11	326.486	.2868
14	5.11	334.020	.2873
14	7.11	347.025	.3051
15	10.11	366.690	.3026
15	12.11	385.952	.3122
15	14.11	393.420	.3121
16	17.11	412.540	.3210

APPENDIX D-4
Raw Data: Tissue Mass (mg)

Controls

	04	08	12	13	18	21	35	38
Heart	825	897	809	945	800	834	1069	1021
Soleus R	63	62	65	73	61	66	60	69
L	57	60	64	72	55	69	60	85
Tibialis } R	394	387	360	418	280	301	373	413
Anterior } L	390	390	352	384	279	338	330	390
Superficial }R	nw	nw	nw	nw	nw	1207	1185	1481
Vastus }L	nw	nw	nw	nw	nw	1140	1268	1637
Epitrochlearis R	57	50	56	69	37	55	60	50
L	68	67	62	52	30	60	54	61

Runners

	10	17	19	23	26	36	39
Heart	1034	983	960	1298	1142	1225	1340
Soleus R	79	72	64	77	72	78	74
L	69	67	66	76	82	74	77
Tibialis }R	382	381	352	372	421	363	387
Anterior }L	404	406	352	340	377	410	369
Superficial }R	nw	nw	1207	1163	1236	1088	1463
Vastus }L	nw	nw	1140	1062	1248	1445	1586
Epitrochlearis R	62	63	52	56	58	57	53
L	66	67	58	63	55	52	64

nw = sample not weighed

APPENDIX D-5
Total Muscle Protein (mg/g)

		Epitrocleasris	Tibialis Anterior
Controls	04	228.26	232.30
	08	216.14	218.67
	12	232.30	247.96
	13	236.85	219.68
	18	215.64	229.78
	21	248.46	247.96
	35	235.84	231.80
	38	206.55	241.90
Runners	10	220.69	240.89
	17		234.32
	19	214.12	245.94
	23	222.71	238.87
	26	240.38	250.48
	36	229.78	238.87
	39	219.17	224.22

APPENDIX D-6
Raw Data: Yield of SR Protein (ug/g)

	light SR	heavy SR
Controls		
	777	825
	752	850
	731	791
	767	838
Training Group		
	765	756
	751	803
	699	746
	742	729

APPENDIX D-7

Raw Data: ATPase activity (umol P_i/mg/min)

1. Ca^{2+} Mg^{2+} ATPase

	light	heavy
Control	3.85	3.56
	3.92	3.21
	3.71	3.43
	3.77	3.28
Runners	3.64	1.99
	3.79	2.17
	3.52	2.10
	3.70	2.34

2. EGTA ATPase

	light	heavy
Control	0.098	0.100
	0.110	0.100
	0.093	0.096
	0.100	0.110
Runners	0.111	0.102
	0.109	0.093
	0.115	0.099
	0.107	0.104

3. NaN_3 ATPase

	light	heavy
Controls	3.88	3.44
	3.79	3.30
	3.90	3.37
		3.40
Runners	3.75	2.08
		1.86
	3.63	1.95
	3.49	2.07

4. RR ATPase

	light	heavy
Controls	3.45	3.22
	3.57	3.45
	3.38	3.30
	3.41	3.38
Runners	3.69	2.40
	3.58	2.17
	3.63	2.28
	3.76	2.33

APPENDIX D-8

"Mass under the curve" for densitometric scans (g)

	ATPase	Calsequestrin	M-55	30 kd
CONTROL-LIGHT	667	38	30	107
CONTROL-HEAVY	609	44	62	155
EXERCISE-LIGHT	523	81	37	82
EXERCISE-HEAVY	518	92	90	229

APPENDIX D-9

Mean body mass (g) for control and training groups at the end of each training week. Mean food consumed (mg·g body weight $^{-1}$) for each week.

	MEAN BODY MASS			MEAN FOOD CONSUMED		
	CONTROL	TRAINING	SIG.	CONTROL	TRAINING	SIG.
WEEK						
1	174.885	176.429	ns	72.762	72.100	ns
2	182.250	174.429	ns	81.450	80.371	ns
3	188.750	186.286	ns	81.575	86.629	ns
4	193.625	193.286	ns	78.500	88.757	p<.001
5	200.750	202.857	ns	77.287	92.700	p<.005
6	208.625	207.857	ns	81.750	97.986	p<.001
7	210.125	211.714	ns	81.300	92.614	p<.001
8	212.500	214.143	ns	75.725	87.643	p<.001
9	216.750	217.429	ns	78.800	93.243	p<.001
10	224.375	221.571	ns	77.550	86.300	p<.005
11	225.375	220.857	ns	76.537	87.086	p<.005
12	230.250	227.714	ns	72.175	87.071	p<.001
13	231.250	223.143	ns	70.762	82.329	p<.005
14	236.500	226.571	ns	67.000	84.786	p<.001
15	237.625	228.857	ns	66.925	90.429	p<.001
16	244.875	237.857	ns	66.050	80.071	p<.001

ns = not significant